activity and reduced toxicity of liposome entrapped doxorubicin

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ACTIVITY AND REDUCED TOXICITY OF LIPOSOME ENTRAPPED DOXORUBICIN

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CONTENTS

LIST OF ABBREVIATIONS

Ara C	: Cytosine arabinoside
Chol	: Cholesterol
DXR	: Doxorubicin
EDTA	: Ethylenediaminetetraacetatic acid
FCM	: Flowcytometer
FU	: 5-Fluorouracil
HDL	: High density lipoprotein
HE	: Hematoxylin and eosin
HPLC	: High performance (or pressure) liquid chromatograph(y)
lip^- DXR	: Doxorubicin encapsulated in negatively charged liposomes
lip^+ DXR	: Doxorubicin encapsulated in positively charged liposomes
LUV	: Large unilamellar vesicle
MCH	: Mean corpuscular hemoglobin
MCHC	: Mean corpuscular hemoglobin concentration
MCV	: Mean corpuscular volume
MLV	: Multilamellar vesicle
MPS	: Mononuclear phagocytic system
MTX	: Methotrexate
PASM	: Periodic acid-silver methenamine
PC	: Phosphatidylcholine
PG	: Phosphatidylglycerol
PS	: Phosphatidylserine
SA	: Stearylamine
SM	: Sphingomyeline
SUV	: Small unilamellar vesicle

Doxorubicin, is one of the most effective cytostatic drugs in clinical practice. Its use, however, is accompanied with considerable toxicity. The toxic effects in the short term are bone marrow depression, nausea and vomiting, alopecia and mucositis. In the long term, treatment with doxorubicin potentially results in cardiomyopathy and clinically overt heart failure.

The interest of the clinician in decreasing the toxicity of this very important drug in cancer medicine, the interest of the Laboratory for Pathology and the Laboratory for Pharmacology of the Dutch National Institute of Public Health and Environmental Hygiene in developing the IgM immunocytoma bearing Lou/M Wsl rat as a model for studying the cytostatic action and toxicity of antitumor drugs, and the interest of the Pharmaceutical Department of the State University of Utrecht in developing the technology of liposomes as drug carriers have been the starting point for the investigations described in this thesis.

Outline of investigation and objectives in this thesis

The principal aim of the study is to determine whether the entrapment of doxorubicin in liposomes results in decreased myocardial damage with preservation of the antitumor effect.

For a better understanding of liposomes as drug carriers, the historical background, the chemistry, and some of the physical aspects and applications of liposomes, of interest for the clinician, are reviewed in Chapter 2.

Treatment with doxorubicin may eventually result in cardiomyopathy. The working mechanisms of doxorubicin are, therefore, given in more detail in Chapter 3. Because oxidative processes elicited by doxorubicin are thought to be responsible for the toxic effects on the heart, the chemistry of oxidative processes elicited by doxorubicin is explained. The relation of these processes to cardiomyopathy and other doxorubicin induced toxicity is discussed. Lines of investigations on possible ways to protect the heart against the detrimental effects of doxorubicin, other than entrapment into liposomes, are mentioned.

In order to correlate and to interprete the results of treatment with doxorubicin entrapped in liposomes measurement of plasma levels and tissue levels were thought to be necessary. Therefore, a method, which enables measurement in microvolumes has been developed (Chapter 4).

The IgM immunocytoma bearing Lou/M Wsl rat is used for the studies in this thesis. This model is described in Chapter 5.

Preliminary studies had shown that male and female Lou/M Wsl rats had different susceptibility for the toxic effects of doxorubicin. Nephropathy in male rats was more severe than in female rats. Therefore a time course study on the development of nephropathy and cardiomyopathy in male and female Lou/M Wsl has been performed, at the same time addressing the question whether the consequences of the nephropathy contribute to the

development of cardiomyopathy (Chapter 6).

Next the effect of encapsulation of doxorubicin in positively and negatively charged liposomes on cardiotoxicity, nephrotoxicity and antitumor activity in comparison to treatment with free doxorubicin has been investigated (Chapter 7).

Because doxorubicin entrapped in negatively charged liposomes appeared to have advantages compared to treatment with free doxorubicin with regard to nephrotoxicity and cardiomyopathy and has been found to have antitumor activity comparable to free doxorubicin, the effect of entrapment of doxorubicin in negatively charged liposomes on suppression of bone marrow activity, which is in daily clinical practice one of the major short term doxorubicin toxicities, has been studied (Chapter 8).

The thesis concludes with Chapter 9 where the results are discussed together with possible lines of future research in animal tumor systems and in clinical practice.

Chapter 2

Introduction to liposomes

2.0 Therapeutic index

The administration of a drug results in several effects, which can be divided into desired and undesired ones. The former justify the application of chemical agents affecting life processes; the latter are defined as toxicity. It belongs to the task of the physician to consider whether his patient will benefit from a drug. In general, the dosage of a drug necessary for its therapeutic effect is required to be several fold lower than for its toxic effects. The ratio between the dose which results in the therapeutic effect, and the dose which elicits toxic effects, is called the therapeutic index.

2.1 Approaches to increased therapeutic index

Most of the currently available antitumor drugs are characterized by a delicate balance between desired effect and toxicity. Whereas in other fields of medicine bone marrow depression, gastrointestinal toxicity, mucositis, alopecia, renal dysfunction, liver cell damage, pulmonary fibrosis, and cardiotoxicity would prohibit the use of a compound, in oncology these side effects are (still) accepted as a necessary evil.

There are several possible lines of research attempts which are used to increase the therapeutic index.

The first approach uses drugs with known efficacy as starting point for attempts to improve the balance between the therapeutic goal and side effects. Chemical engineering on existing drugs results in so called analoga; these are specially designed, preferably using knowledge about structure-activity relationships. Doxorubicin, a structural analog of daunorubicin, was discovered after selecting a mutant of the daunorubicin producing microorganism. Nowadays, quite a family of rationally designed analogs, e.g. 4'-epidoxorubicin, deoxydoxorubicin, and 4-demethoxydaunorubicin, is entering clinical trials (Casazza 1978).

The second possibility to increase the therapeutic index is brought about by alternative routes of administration. For example, 5-fluorouracil (Ansfield, 1976; Grage 1979), doxorubicin (Haskell, 1974), cisplatin (Calvo, 1980), and methotrexate (Carter, 1977) have been administered intraarterially instead of intravenously in order to obtain high levels of cytostatic drug in tumor tissue. Intraperitoneal administration of a cytostatic drug is based on the pharmacokinetic rationale that an intraperitoneally localized tumor may be exposed to very high local drug concentrations, while at the utmost systemic levels equal those normally encountered in intravenous therapy. Currently, the intraperitoneal administration of cisplatin is being studied for ovarian cancer (Howell, 1982; 1984; McVie, 1985).

The third way to increase the therapeutic index is based on the application of knowledge about the mechanism of drug activity, pharmacokinetics, and cell kinetics, resulting in a rationally designed dosage schedule. Continuous infusion of S-phase specific drugs, e.g. methotrexate and cytosine arabinoside, may increase therapeutic effectiveness, because more of the dividing cells, reach the S-phase, and are exposed to the steady state serum drug level.

Drug carriers are the fourth method for increasing the therapeutic index in cancer chemotherapy. In particular, liposomes as drug carriers have been the subject of study on their possible role in modifying drug behavior (Weinstein, 1984; Weinstein and Leserman, 1984).

The theoretical arguments for their use are: 1) prolongation of drug effect due to prolonged circulation of encapsulated drug; 2) targeting of liposomes into particular organs by their physical properties; 3) targeting of liposomes by attaching an antibody or other ligand; 4) targeting of liposomes to the mononuclear phagocytic system (MPS); 5) confinement of intact liposomes to an anatomical compartment, e.g. the peritoneal cavity; 6) protection of a drug from metabolism and immune attack; 7) reduction of drug toxicity in tissues which do not accumulate liposomes; 8) the physical properties of liposomes may result in the selective local release of drug by controlling local temperature or pH; 9)

bypassing cell membrane related drug transport systems.

2.2. Liposomes: Definition and historical background

Liposomes are microscopic structures consisting of one or more concentric lipid bilayers enclosing an equal number of aqueous spaces (Weinstein and Leserman, 1984).

The discovery of the fundamental structure of biological membranes and the availability of phospholipids have led Bangham and coworkers (1964) to investigate the effects of hydration of dry purified phospholipids of cellular origin (Bangham, 1964). They demonstrated that the structures which were formed by hydration of the aforementioned phospholipids were compatible with closed systems, because they behaved like osmometers. In addition, the bilayer structure of the leaflet has been shown, which separates each aqueous compartment from its neighbour (Bangham, 1964, 1967). Initially, these structures were used for the fundamental research on membrane structure. Later on, the ability to entrap water soluble or lipophilic compounds into the aqueous or phospholipid compartments respectively, gave rise to the idea to use liposomes as drug carriers.

2.3. Chemistry and physical properties

Lipids consist of a hydrophilic headgroup and a lipophilic hydrocarbon tail. The degree of water solubility determines the behavior of lipids in water or in aqueous solutions: preponderance of the headgroup gives rise to micelles, in which the hydrocarbon chains extend inward and the head groups outward (Weinstein and Leserman 1984); preponderance of the hydrocarbon tails results in structures in which the head groups face an aqueous center (inverted micelles), or prevents hydration at all. The case where a balance between the hydrophilic head group and the hydrophobic hydrocarbon chains occurs, bilayers are formed, in which the hydrophobic chains face each other and the hydrophilic head groups face outwards in the aqueous phase. The lipids which form bilayers have characteristically two hydrocarbon chains. Such lipids are: phosphatidylcholines (more commonly named lecithin) (PC), phosphatidylglycerols (PG), phosphatidylserines (PS),

sphingomyelines (SM), and phosphatidylethanolamines. The lipids are classified by their head group, whereas in each class the length and the degree of saturation of the hydrocarbon chain varies.

The electrical charge of bilayer membranes is defined in vitro at pH 7. One discerns neutral, negatively and positively charged bilayer membranes. A negative charge is provided by incorporation of phosphatidylserine, phosphatidylinositol, phosphatidic acid, phosphatidylglycerol, or dicetylphosphate. Incorporation of stearylamine provides a positive charge.

Each hydrated pure lipid species is characterized by a liquid-crystalline transition temperature, at which the lipid chains undergo an endothermic change in state from a quasi-crystalline order to one in which the alkyl chains have more motional freedom. The permeability of phospholipid membranes has been shown to increase at transition temperature (Blok, 1976). Designing liposomes, consisting of lipids with transition temperatures a few degrees above the physiological temperature, offers the possibility to induce release of encapsulated drugs in selectively heated parts of the body.

2.4. Methods of preparation

Liposomes arise when dry phospholipids are brought into contact with water. The classical technique to prepare liposomes is as follows. Phospholipids are dissolved in an organic solvent and put into a pear shaped flask. Under continuous rotation the organic solvent is evaporated, resulting in a dry phospholipid film on the wall of the flask. Thereafter, the phospholipid film, which should be as thin as possible, is hydrated in an aqueous buffered solution under shaking. This procedure results in multilamellar vesicles (MLV), i.e. vesicles consisting of two or more bilayers separated by interlamellar spaces filled with water (Fig. 1). The size distribution is quite heterogeneous and varies from several hundreds of nanometers to several micrometers in diameter. The size distribution can be narrowed down by stepwise extrusion through Nucleopore polycarbonate membranes with defined pore sizes (Olson, 1979). This procedure narrows the particle size distribution by removing particles with diameters exceeding the pore size.

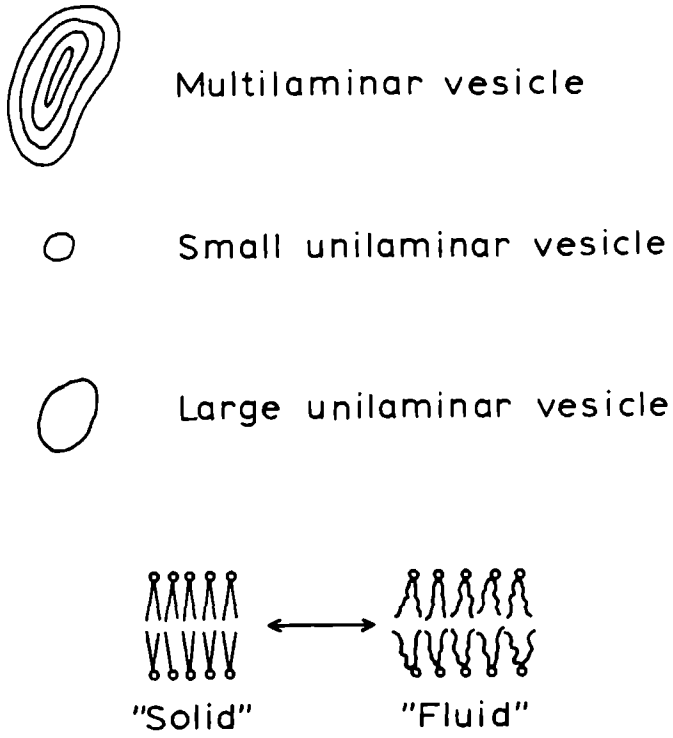

Fig. 1. In the upper part of this figure a schematic representation of the different types of liposomes is given. Each solid line represents a bilayer. In the lower part the bilayer is shown in detail, and the transition from the solid state into the fluid state is suggested.

The lower region of particle distribution can be controlled by dialysis over polycarbonate membranes with defined pore sizes (Bosworth, 1982). The average size of lipid vesicles dispersed in water can be determined by dynamic light scattering and turbidity

measurements (Chong, 1976).

It is possible to prepare liposomes, which consist of unilamellar vesicles. These are divided into two groups: small unilamellar vesicles (SUV) with a diameter of < 100 nm and large unilamellar vesicles (LUV) with a diameter of > 100 nm. SUV can be prepared from dispersions of MLV by sonication, whereafter they are freed from MLV by column chromatography.

LUV are prepared by injecting phospholipids dissolved in ether into the aqueous phase which is warmed above the boiling point of ether (Deamer, 1978). The ether vaporizes upon contacting the aqueous phase, and the dispersed lipids form unilamellar liposomes. A draw-back of this technique is the inability to incorporate macromolecules and compounds susceptible for ether. In addition, the resulting dispersions are not concentrated.

A second technique to prepare LUV is known as "the reverse phase evaporation technique" and has been described by Szoka and Papahadjopoulos (1978). An aqueous buffer is dispersed into a solution of phospholipid(s) in an organic solvent. It is suggested that inverted micelles are formed. The organic solvent is removed by evaporation under reduced pressure. In the last stage of the procedure inverted micelles desintegrate and form unilamellar vesicles. Encapsulation efficiency is high and macromolecules can be easily entrapped into the aqueous phase.

Another method for preparing LUV has been described by Papahadjopoulos (1975, 1978). It is based on the calcium-induced fusion of sonicated phosphatidylserine (PS) vesicles and subsequent chelation of calcium by EDTA. This procedure results in the incorporation of macromolecules with high efficiency.

The efficiency of water volume trapping varies among the different types of liposomes, and is expressed in ml/mmol. For SUV this value is about 0.5 ml/mmol; for MLV 2.5 ml/mmol; for LUV 7 ml/mmol; and for LUV prepared by the reverse phase evaporation technique 14 ml/mmol (Papahadjopoulos, 1978).

2.5. Interactions of liposomes with plasma proteins

Experiments in vivo had shown that some liposomes loose their contents very rapidly after intravenous injection. In vitro

analysis of this phenomenon has demonstrated that liposomes are attacked by high density lipoprotein (HDL). Transfer of liposomal PC into the HDL complex has been shown. In addition, small amounts of phospholipids have also been found in very low- and low density lipoproteins (VLDL and LDL), probably as a result of phospholipid transfer from the HDL complex.

The susceptibility to HDL attack by vortex-dispersed liposomes appears to be maximal at the gel to liquid crystalline phase transition temperature. This phenomenon can be used for hyperthermic targeting of liposomes. Sonicated liposomes do not show such a distinct temperature optimum, which is ascribed to the decreased radius of curvature after sonication. Enzymatic activity of HDL is hampered by a small radius of curvature.

The incorporation of cholesterol in the bilayer confers structural stability on the liposome. Cholesterol not only diminishes the diffusion of a variety of solutes through the bilayer membrane, it also reduces the susceptibility towards phospholipase action (Weinstein 1980, Yatvin 1981).

Human serum albumin is known to be adsorbed onto the outer surface of liposomes. Such human albumin-coated liposomes evoke a strong immune response in rabbits, leading to the rapid formation of high anti-human serum albumin titers. A detrimental effect on the integrity of liposomes has not been proven for the adsorption of albumin to liposomes. Albumin, as well as alpha-, beta- and gammaglobulins in vitro have been shown to interfere with liposome-cell interaction. The implications of these in vitro observations for the in vivo behavior of liposomes are not clear.

A concise review on the interaction of liposomes with plasma proteins is given by Scherphof et al. (1981).

2.6. Theoretical considerations on liposome cell interaction

Cells can interact with liposomes in four different ways (Pagano, 1978) :

1) Stable adsorption
2) Endocytosis
3) Fusion
4) Lipid transfer/exchange

The possible mechanisms are schematically represented in fig. 2.

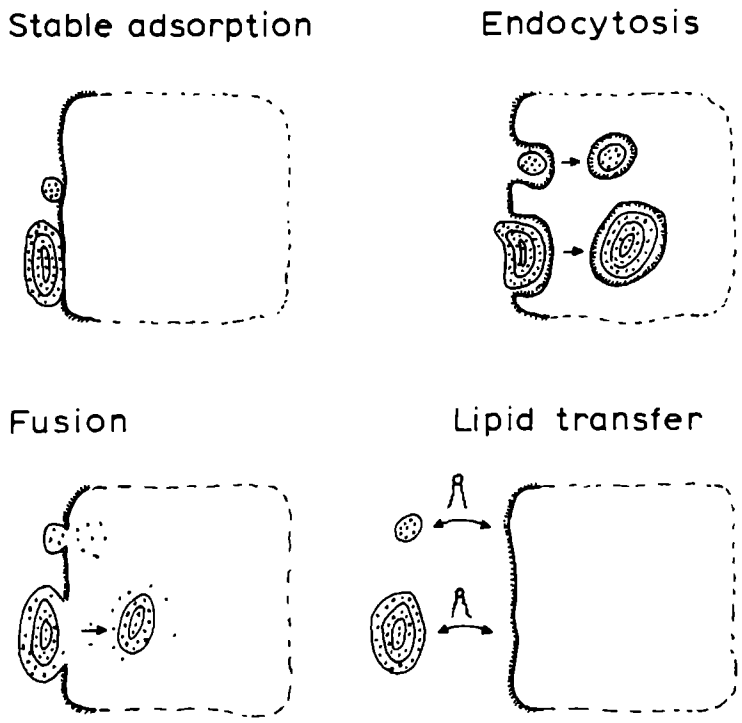

Fig. 2. Schematic representation of liposome-cell interaction (after Pagano 1978).

1. Stable adsorption is the association of intact liposomes with the cell surface without their internalization. The adsorption may be mediated by nonspecific forces, e.g. electrostatic forces or by specific components (e.g. surface receptors, antibodies).

2. Endocytosis is the uptake of intact liposomes into endocytotic vesicles. In general, endocytosis leads to delivery to the lysosomal apparatus.

3. Fusion is the merging of the lipid bilayer (the outermost bilayer of a MLV) with the plasma membrane and concomitant release of liposome contents into the cytoplasmic space. Leakage of liposome contents into the medium during the fusion event is possible. Secondary processes may concentrate the vesicle contents

from the cytoplasma within other intracellular compartments (e.g. lysosomes). In the case of fusion of a MLV with a cell, a multilamellar form with one bilayer less than the original should be found in the cytoplasm.

4. Lipid transfer/exchange is the transfer of lipid molecules between liposomes and cells without cell association of aqueous liposome contents.

Experimental criteria for distinguishing various mechanisms of liposome-cell interaction have been described by Pagano (1978).

2.7. Liposome-cell interactions in vitro

The uptake and processing of liposomes by rat liver macrophages have been extensively studied in vitro in chemically defined media (Dijkstra, 1983). Endocytosis was found to be the predominant uptake mechanism for negatively charged LUV and neutral vesicles, whereas about one half of positively charged vesicles remained adsorbed at the cell surface. The attachment of liposomes appeared to be dependent on the presence of divalent cations, in particular Ca^{++}. Clues have been found that the binding site on the Kupffer cell is identical for positively charged and neutral liposomes, and differs from the binding site for negatively charged liposomes. After endocytosis of negatively charged LUV the radioactive marker of the aqueous compartment appeared in the incubation medium almost completely, whereas only 30% of the radioactive marker of the lipid compartment was released into the incubation medium. These effects were inhibited by ammonia and chloroquine, inhibitors of lysosome activity. After intralysosomal degradation of liposomes endocytosed by Kupffer cells the choline moiety of phosphatidylcholine or sphingomyelin was found to be reutilized for de novo synthesis of cellular phosphatidylcholine.

Human leukocytes have shown to take up MLV in whole blood in vitro (Kuhn, 1983). Human peripheral blood monocytes, cultured in vitro, take up liposomes intactly, negatively charged ones more rapidly than positively charged or neutral vesicles (Mehta, 1982).

2.8. Clearance and distribution of liposomes in vivo

Size and surface charge of liposomes are two of the major

determinants of liposome clearance (Juliano, 1981). SUV with diameters of 20-50 nm have been found to persist longer in the circulation than MLV with diameters of ca. 1000 nm of the same composition. The clearance rate of liposome samples, homogeneous in size, can be described by a simple exponential function, whereas the clearance of heterogeneous samples can only be fitted by a sum of exponentials. Negatively charged SUV are more rapidly cleared from the circulation than neutral and positively charged ones.

Intravenously administered liposomes have been reported to be removed from the blood predominantly by liver and spleen. In the liver large liposomes (> 200 nm) have been found to be almost exclusively associated with Kupffer cells (Segal, 1974; Roerdink, 1981; Poste, 1982). In contrast, liposomes with diameters of 30-100 nm also have been found in hepatocytes, probably because they are able to pass the fenestrae in the endothelial lining of sinusoids (Scherphof, 1983). In vivo, ^{111}In-labelled bleomycin, encapsulated in liposomes, injected intravenously in two patients was found to accumulate in the liver, as determined by whole-body scanning. This radioactivity was found to be concentrated in hepatic lysosomes as has been shown in studies on percutaneous liver biopsies, taken 90 min. after injection (Segal 1975).

2.9. Cytostatics and liposomes

2.9.1. Incorporation of drugs

The association of drugs with liposomes is determined by the oil-water partition coefficient and by charge (Juliano, 1979). Water-soluble drugs are entrapped in the aqueous compartments by forming liposomes with an aqueous phase containing dissolved drugs. The entrapment efficacy for small molecules approaches the ratio between the volume of entrapped aqueous phase and the original volume of the aqueous phase. Lipophilic drugs are incorporated into the bilayer. Most drugs are amphophilic, resulting in partition between the aqueous compartment and the bilayer.

2.9.2. Methotrexate

In the mid-seventies the encapsulation of methotrexate (MTX)

in liposomes has been studied. Because at pH 7.4 MTX is negatively charged, liposomes with a positive charge in order to get an electrical interaction between MTX and the liposome were used (Kimelberg 1976, 1978). A single i.v. injection of MTX entrapped in liposomes in Macacus irus resulted in prolonged MTX levels at 4 hours after administration, compared to free drug. The plasma level after i.v. injection of MTX in mechanically disrupted liposomes was 6-fold higher at 4 hours than after free MTX, whereas the plasma level after administration of sonically disrupted MTX containing liposomes was 100-fold compared to the plasma level after injection of free drug, pointing at the particle size as a determinant of in vivo behavior. In parallel with these findings the renal excretion of MTX entrapped in mechanically disrupted liposomes was 18% of the renal excretion of MTX after the same dose of free MTX, whereas after administration of MTX entrapped in sonically disrupted liposomes the renal MTX excretion was 1%. There was more than a 160-fold increased uptake of MTX in the spleen after administration of mechanically disrupted MTX containing liposomes. For sonically disrupted liposomes this ratio was 20. The next high tissue levels were found in the liver. Compared to intestinal tissue MTX levels after administration of free drug, the levels in intestinal tissue after administration of liposome entrapped MTX were low. Similar results were obtained by Colley (1975) and Freise et al. (1977).

With regard to antitumor effect entrapment of MTX in liposomes showed no better results compared to treatment with free drug (Kimelberg 1978). Other investigators have found increased efficacy of liposome entrapped MTX against sensitive and MTX insensitive tumors (Kosloski 1978, Kaye 1981, Richardson 1982).

2.9.3. Cytosine arabinoside

Entrapment of cytosine arabinoside (Ara-C) in liposomes enhanced the in vitro cytotoxic effect against L1210 cells (Mayhew 1978). This was only found in the case of positively charged vesicles. Liposome entrapped Ara-C prolonged survival of mice, intraperitoneally inoculated with L1210 (Kobayashi 1975, 1977). In parallel with the in vitro studies Kataoka (1978) found an

advantage for positively charged vesicles. Mayhew (1978) found no difference with regard to charge, however MLV appeared more effective than SUV. Entrapment of Ara-C in liposomes resulted in prolonged plasma Ara-C levels, and a preferential uptake in the liver (Mayhew 1978). The enhancement of the therapeutic effect of liposome entrapped Ara-C is thought to be due to protection against biodegradation.

Larger MLV resulted in increased retention of Ara-C in the lung (Hunt 1979). Intratracheal instillation of Ara-C entrapped in liposomes confined the inhibition of the incorporation of ^{3}H-thymidine only into pulmonary tissue (McCullough 1979).

2.9.4. Actinomycin-D

Entrapment of actinomycin-D in liposomes rendered drug-resistant tumor cells *in vitro* susceptible for this drug by enhancement of drug uptake (Poste 1976). Treatment of tumor bearing mice with liposome encapsulated actinomycin-D prolonged their survival compared to treatment with equal dose of the free drug (Gregoriadis 1975). In addition, administration of liposome entrapped actinomycin-D appeared less toxic to mice (Rahman 1974). Actinomycin-D entrapped in the lipid phase of liposomes resulted in high actinomycin-D levels in the lungs and low levels in the intestinal wall, whereas free actinomycin-D and actinomycin-D entrapped in the aqueous phase showed high levels in the intestinal wall and low levels in the lungs (Rahman 1975).

2.10. Non cytostatic drugs and liposomes

2.10.1. Insulin

In order to circumvent the inconveniences of daily insulin injections, the oral administration of insulin entrapped in liposomes has been studied. In diabetic rats orally administered insulin entrapped in liposomes lowered serum glucose levels whereas non-encapsulated insulin did not (Patel 1976, Tragl 1979). Immune reactive insulin ^{125}I-insulin could be detected in serum after oral administration of ^{125}I-insulin in liposomes (Arrieta-Molera 1982). Similar results were obtained in rabbits (Arrieta-Molera 1976). However, disappointing results were obtained by Shenfield (1982).

Administration of liposome entrapped insulin to normal man did not result in a significant change in blood glucose concentration (Patel 1978).

Instability of insulin containing liposome preparations and unpredictable dose response relationship appear to be the major impedements for further development of this line of investigation.

2.10.2. Enzyme replacement

The treatment of enzyme deficiencies with free enzyme encounters the problem of immunological effects, inactivation in plasma and interactions with substrates in plasma or on cell surfaces of blood elements or tissues. Entrapment of enzymes in liposomes is thought to bypass these problems. Uptake of exogenous horse-radish peroxidase in phagocytes of Mustelus canis has been accomplished by immunoglobulin-coated liposomes (G.Weissmann, 1975, 1976). Injection of neuraminidase entrapped in liposomes prevented the action of this enzyme on substrates in plasma and on blood cells, and most of it was recovered from the lysosomal fraction of the liver (Gregoriadis, 1974). In vitro treatment of fibroblasts of feline G_{M1} gangliosidosis with B-galactosidase encapsulated in liposomes resulted in increased B-galactosidase activity and increased clearance of glycopeptides, compared with treatment of non-entrapped B-galactosidase (Reynolds, 1978). In vitro it has been possible to introduce purified placental hexosaminidase A into polymorphonuclear leukocytes of Tay-Sachs patients by means of immunoglobulin-coated liposomes (Cohen, 1976). In clinical medicine liposome-entrapped glucocerebroside B-glucosidase has been used in Gaucher disease without a convincing clinical response (Gregoriadis, 1982).

2.10.3. Miscellaneous applications

Treatment of mice with liposome-encapsulated Amphotericin B did not result in abnormalities in blood chemistry and histology and a LD50 could not be obtained, in contrast to treatment with free Amphotericin B. The encapsulated drug was as effective as free drug in the treatment of Candida albicans, altogether resulting in an improved therapeutic index (Lopez-Berestein, 1983).

Entrapment of drugs in liposomes has been proposed for the enhancement of local drug concentrations and the restriction of drug effects to the area to be treated in topical drug applications in ophthalmology and dermatology (Schaeffer, 1982, Mezei, 1982). The application of liposome entrapped triamcinolone acetonide to rabbit skin resulted in higher triamcinolone acetonide concentrations in epidermis and dermis and lower concentrations in blood when compared with the classical way of application. Lectin-mediated attachment of liposomes to cornea enhanced the transcorneal flux of entrapped carbachol across isolated rabbit corneas under conditions of continuous tear flow.

The encapsulation of hemoglobin in liposomes has been suggested in order to circumvent incompatibilities in blood group serology (Gaber, 1983).

Succesful removal of iron and plutonium has been described after administration of metal chelators entrapped in liposomes in mice (Rahman, 1975b, Rosenthal, 1975, Lau, 1983).

2.11. Immunological aspects

The phospholipids used in the majority of studies on liposomes encapsulating anticancer drugs are usually thought to be of low antigenicity. The administration of N-dinitrophenyl phosphatidylethanol-amines inserted into liposomal membranes has been shown to raise antibodies (IgM and IgG) in the guinea pig (Uemara 1975). In addition, glycolipids, lipid A (the lipid component of bacterial lipopolysaccharide), and several anionic phosphoglycerides have shown antigenicity when injected as constituents of liposomes (Alving 1977). Furthermore, rabbits develop antibodies not only against lipid A but also against phosphatidylcholine, when injected with lipid A inserted liposomes composed of phosphatidylcholine (Schuster, 1979).

Not only insertion of antigens into the bilayer membrane, but also entrapment of antigen in the aqueous phase in liposomes or binding to liposomal surface gives rise to antibodies (van Rooyen 1977, 1983). Liposomes have been suggested as adjuvants for vaccination.

Positively charged liposomes, containing dimyristoyl

phosphatidylcholine, cholesterol and certain glycolipids, have been shown to activate the alternative complement pathway *in vitro* (Cunningham 1979).

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Introduction to doxorubicin

3.1. Origin and chemistry

Doxorubicin belongs to the anthracyclines, members of the rhodomycin group, which are produced by Streptomyces species. Doxorubicin is extracted with a mixture of acetone and aqueous sulfuric acid from colonies of Streptomyces peucetius var. caesius (Arcamone 1969). Doxorubicin (= 14-hydroxy-daunorubicin) might be considered as an analog of daunorubicin.

Anthracyclines are composed of a tetracyclic chromophore linked to a sugar. The general structure is shown in fig. 1. The anthracycline structure is responsible for their behavior as fluorescent compounds.

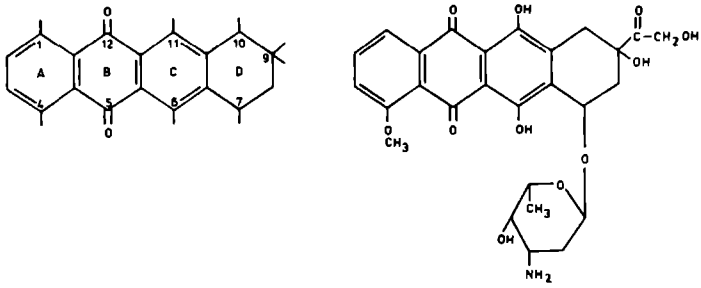

Fig. 1. On the left side the general structure of anthracyclines is shown. The right side represents the structure of doxorubicin.

3.2. Analytical methods

The fluorescent characteristics of these compounds have enabled the development of relatively simple assay methods. The measurement of total fluorescence of an extract of tissue or plasma was the first technique used (Schwartz 1973). The development of thin-layer chromatography enabled the detection of several

metabolites (Chan 1979). Adequate separation of metabolites is also a feature of high performance liquid chromatography, which is nowadays commonly used because it is less expensive and less laborious than thin layer chromatography (Israel 1978, Eksborg 1979, Oosterbaan 1984). The cellular uptake of doxorubicin has been studied by flow cytometry (Sonneveld 1981, Durand 1981, 1981a, Speth 1985).

3.3. Pharmacokinetics

Plasma doxorubicin clearance curves after intravenous bolus administration are triphasic, with half-lives of 8-25 minutes, 1.5-10.0 hours, and 24-48 hours, respectively (Myers 1982). Doxorubicin binds to a considerable extent to plasma proteins and to tissue sites. The major metabolite, among others, is doxorubicinol, which also has some antitumor activity. Doxorubicin is metabolized to doxorubicinol predominantly in the liver by aldo-ketoreductases. These enzymes are also present in peripheral blood cells which has implications for the handling of blood and plasma samples (Eksborg 1981). Biliary excretion of doxorubicin is the most important way of elimination. The renal excretion of doxorubin in man has been found to be insignificant (Benjamin 1973).

3.4. Range of activity

Doxorubicin is one of the major drugs in clinical oncology. It is active against acute leukemias, malignant lymphomas, breast cancer, sarcomas, ovarian cancer, gastric cancer, and small cell lung cancer. In addition, doxorubicin is instilled locally in the urinary bladder for the treatment of superficial bladder cancer.

3.5. Mechanisms of action

Four biochemical events are thought to play a role in the biological effects of doxorubicin. These are:

1. DNA binding
2. free radical formation
3. metal chelation
4. membrane effects.

The relationship between these events is very complex and the contribution to the eventual biological effects is not known for each event separately. A detailed review of the biochemical background and mechanisms is beyond the scope of this thesis. For a detailed and updated review of this complicated, but very exciting matter, the reader is referred to Myers (1979, 1980, 1981, 1982, 1982a, 1983, 1984).

The major points will be reviewed in the following paragraphs.

3.5.1. DNA binding

Doxorubicin intercalates DNA with subsequent effects on DNA and RNA synthesis and DNA damage. The tetracyclic chromophore inserts itself between base pairs perpendicular to the long axis of the double helix, the major interaction coming between the B and C rings of doxorubicin and the bases above and below them. Cells have been shown to proliferate from G1- through S- to G2-phase despite the presence of doxorubicin.

3.5.2. Free radical formation

A free radical is a compound with one unpaired electron. In the presence of NADPH, microsomal P450 reduces doxorubicin to its semiquinone radical. In the presence of oxygen the semiquinone radical transfers the unpaired electron to yield the superoxide radical. This radical is dismutated to hydrogen peroxide, which subsequently reacts with superoxide to form the hydroxyl radical ($OH^{\cdot}$). In overview:

(1) $2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$

(2) $O_2^- + H_2O_2 \longrightarrow OH^{\cdot} + OH^- + O_2$

The hydroxyl radical is one of the most reactive substances known and is able to attack pyrimidine and purine bases, thiols and amines. The superoxide radical damages cell membranes and causes cleavage of DNA.

3.5.3. Metal ion chelation

Doxorubicin chelates zinc, copper, and iron. In particular the chelation of iron seems to have major implications. Equation (2), described in the former paragraph is thought to be catalyzed via the following mechanism:

(3) $O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$

(4) $Fe^{2+} + H_2O_2 \longrightarrow OH^{\cdot} + OH^- + Fe^{3+}$

So, the transfer of electrons is enhanced in the presence of iron or its chelate.

3.5.4. Membrane effects

Doxorubicin has been found to bind to erythrocyte membranes and to cause altered membrane morphology and lysis. Of all cell membrane phospholipids, doxorubicin binds most tightly to cardiolipin. The highest cardiolipin concentrations are found in mitochondrial membranes, in particular the inner leaflet.

3.6. Toxicity

3.6.1. Cardiotoxicity

Soon after the introduction of daunorubicin into the treatment of acute leukemias of childhood, it became apparent that this drug was associated with the development of heart failure. Subsequently, also doxorubicin, which has a wider range of application in comparison to daunorubicin, appeared to induce myocardial damage. The incidence of clinical overt heart failure has been found to increase sharply above a cumulative dose of 550 mg/m^2. An increased incidence of anthracycline-induced cardiomyopathy has been identified after radiation therapy of the mediastinum and in patients with hypertension.

Clinically, anthracycline-induced cardiomyopathy is characterized by forward and backward failure. Although improvement of heart failure has been observed, the prognosis is, in general, bad: the mortality rate can be as high as 50%.

Free radical formation with subsequent oxidative damage is thought to be the major mechanism of cardiomyopathy. In particular, because the cardiac tissue of some species has been shown to lack catalase, which eliminates hydrogen peroxide. In addition, doxorubicin destroys glutathione peroxidase (Doroshow 1980, Revis 1978). As a consequence, two major enzymes involved in the detoxification of hydrogen peroxide, are lacking in the heart, at the very moment that the production of hydrogen peroxide is stimulated by doxorubicin.

The membrane effects, described earlier, may result in membrane damage, which has detrimental effects on calcium-handling. In the heart muscle contractility force is regulated by governing Ca^{2+} concentrations.

Although in the past decennium many, often very sophisticated, studies on the biochemical backgrounds of anthracycline toxicity have been performed, a clear picture has not yet evolved.

3.6.2. Other toxicities

Myelosuppression is the dose limiting toxicity of doxorubicin in everyday practice. Leukocyte and thrombocyte count reach their nadir 7 to 10 days after administration, and their counts return to normal levels within 21 days after injection.

Alopecia is an unevitable consequence of treatment with doxorubicin. Rarely, mucositis is encountered.

In contrast to cardiotoxicity, the other toxic events can readily be explained by the inhibition of the proliferative activity of bone marrow, hair root, and gastrointestinal mucosa.

3.6.3. Lines of investigation on cardioprotection

Because there were strong indications that oxidative damage was the principal cause of cardiomyopathy, early in anthracycline research attempts have been made to prevent cardiomyopathy by anti-oxidants or "radical-scavengers" (Myers 1977). Among these are alpha-tocopherol, N-acetylcysteine and carnitine (Sonneveld 1978, Wang 1980, Van Vleet 1980, Mimnaugh 1979, Breed 1980, Freeman 1980). No consistent results have been obtained.

With regard to the role of iron as a catalyst in the oxidative

avalanche the possible role of EDTA-derived chelating compounds ICRF 187 and ICRF 159 as protector have been investigated (Herman 1981, Herman 1983, Guilani 1981).

The development of analoga is another line of research (Arcamone 1985).

For the clinician variations of the dosage schedule are the obvious way to make an attempt to avoid cardiotoxicity (Weiss 1976, Chlebowski 1980, Legha 1982, 1982a).

The entrapment of doxorubicin in liposomes has resulted in reduction of cardiotoxicity in animal models (Rahman 1980, 1981, Olson 1982, Gabizon 1982, Forssen 1981, 1983).

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Chapter 4

ANALYSIS OF ADRIAMYCIN AND ADRIAMYCINOL IN MICRO VOLUMES OF RAT PLASMA

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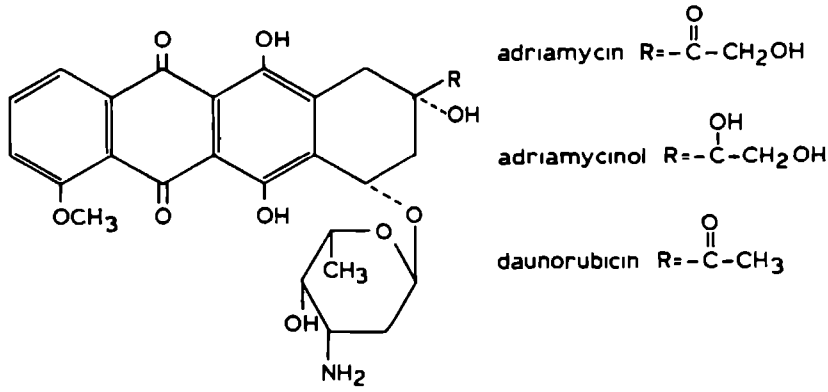
Despite ten years of intensive investigation into the therapeutic effects and side-effects of the anthracycline derivative adriamycin, cardiac toxicity is still one of its most severe toxic effects. This limits the dose and consequently its application in cancer chemotherapy. As part of an investigation of the cardiac toxicity caused by adriamycin in tumor-bearing rats, pharmacokinetic data were needed to find out whether there is a correlation between histological changes of heart tissue, the dose of adriamycin and the method of administration. Hence, an assay for adriamycin and its active metabolite adriamycinol was needed to establish pharmacokinetic parameters, such as peak levels, rate of distribution and elimination. If several rats are used for one test, the results due to interindividual differences will not be reliable. Therefore, we decided to establish pharmacokinetic parameters for one single rat.

From the literature (1, 2) one can conclude that after the initial distribution phase, a biphasic disappearance pattern is to be expected. Therefore, 10-15 plasma samples are needed to obtain a complete plasma concentration disappearance curve. This limits the volume of the plasma samples to be analyzed to a maximum of 100 ul (the total blood volume of a rat is 4-6 ml).

Recently described high-performance liquid chromatographic methods

that involve fluorescence detection for selective determination of adriamycin and adriamycinol, are preferred to non-selective methods like total fluorescence analysis (3,4) and radio-immunoassays (4, 5).

Reversed-phase (6-11) as well as straight-phase (12-14) methods can be used. With straight-phase liquid chromatography and gradient elution, one can analyse nearly all the metabolites, including the inactive aglycones, glucuronated and sulphonated metabolites. Reversed-phase liquid chromatography can easily be applied for the selective assay of adriamycin and its active metabolite adriamycinol. If these methods are to be applied successfully in pharmacological practice, further requirements are easy sample treatment and simple apparatus; gradient elution techniques, for instance, should be omitted if possible.



In this paper, a simple and selective high-performance liquid chromatographic method involving fluorescence detection is described. Rat plasma samples each with a volume of 100 ul were used, plasma levels being within the therapeutic range.

To test the applicability of this method in pharmacological practice, plasma disappearance curves and preliminary pharmacokinetic data for several rats have been determined. A typical plasma concentration-time curve and related pharmacokinetic data are presented.

Chemicals and apparatus

Adriamycin-HCl was obtained from Farmitalia in vials of 10 mg with 50 mg lactose. Farmitalia kindly gave us samples of adriamycinol. Daunorubicin-HCl was obtained from Specia in vials of 20 mg with 100 mg D-mannitol. Chromatographic solvents and other chemicals were of analytical grade and were used without further purification. The chromatographic system consisted of a Model 6000A solvent delivery system and a U6K septumless injection system (both from Waters Assoc., Milford, MA, U.S.A.), and a Perkin-Elmer Model 204 fluorescence detector supplied with a Hellma 25-µl flow cell (Hellma, type 176.70). A Merck LiChrosorb RP-8 (50432 C8) reversed-phase column (125 x 4 mm I.D., particle size 5 µm) was used. The mobile phase consisted of acetonitrile-distilled water-0.1 M H_3PO_4 (31:61:8) containing 10 µg desipramine-HCl per ml (pH 2.3), and was filtered through a 0.2-µm filter and deaerated ultrasonically before use. The flow-rate was 1.5-2 ml min^{-1}. Fluorimetric detection was performed at excitation wavelength 470 nm and emission wavelength 565 nm. Quantitation was based on peak height ratios using the structural analogue daunorubicin as an internal standard. The chromatographic analyses were performed at ambient temperature.

Sample pre-treatment of micro-volumes of rat plasma

The rats received i.v. injection of adriamycin. By cannulating the vena jugularis, heparinized blood samples of about 200 ul were collected in polypropylene tubes over a period of 3 days. The samples were centrifuged immediately and stored at -20°C prior to analysis. For the extraction of adriamycin and adriamycinol, 100 µl plasma was mixed with 100 µl borate buffer pH 9.0 in a conical polypropylene tube of 1.5 ml. The buffer was composed of 24.7 g boric acid, 6.8 g sodium hydroxide, 29.7 g potassium chloride and 10 mg desipramine-HCl per litre (7, 15). Appropriate amounts of the internal standard daunorubicin were added in 10-µl volumes of an aqueous solution. A 0.5-ml volume of a chloroform-1-heptanol mixture (1:1) was added. The mixture was vortexed for 45 sec and centrifuged for 5 min at 2500 g. The aqueous upper layer was removed and the organic layer was transferred to a new conical polypropylene tube of 1.5 ml containing 100 µl 0.2 M phosphoric acid. After 1 min vortexing and 5 min centrifugation at

2500 g, 10 to 90 µl of the aqueous phase, depending on the expected concentration level, was injected into the chromatographic system. All the glassware used was silanized before use by treating it with a solution of 2% trimethylchlorosilane in toluene, followed by a washing procedure with methanol.

Blood sampling

Male Wistar rats all weighing about 250 to 300 g were cannulated in the left vena jugularis. The cannule was flushed with a heparin solution twice a day and after each blood sampling. Adriamycin (2 $mg.kg^{-1}$) was administered by i.v. bolus injection. The valve of the cannule was opened and about 200 µl blood was collected in heparinized polypropylene tubes. If the rats damaged the cannule, blood was collected by orbital puncture.

Calculation

The plasma levels of adriamycin were analysed using the HP 9810, programmed with the Wagner stripping method (16).

The straight-phase liquid chromatographic method for the analysis of adriamycin and adriamycinol, described by Baurain et al. (14), is the only one that uses 100 µl of plasma. However, the authors showed its applicability in the µg range only. All other methods mentioned earlier in this paper, reversed-phase as well as straight phase, need 1-4 ml of plasma.

To determine adriamycin and adriamycinol in the ng range, with acceptable accuracy, using 100 µl of plasma, special techniques have to be developed to prevent loss of adriamycin and adriamycinol, because of their strong adsorptive properties. The flexibility of the sample pre-treatment is limited by the adsorptive properties. Polypropylene tubes should be used if possible; if glassware is used it should all be silanized (17). However, even with silanized or siliconized glassware, adriamycin may still be adsorbed from an aqueous solution. Desipramine-HCl, with comparable adsorptive properties, was added to the buffer in the extraction procedure and to the mobile phase in the chromatographic procedure, in order to decrease the number of active, adsorptive sites. The syringe was pre-treated with trimethylchlorosilane for the same reason and had to be washed with a 4M hydrochloric acid-methanol mixture (1:9). The syringe was washed many times with water between each injection, to prevent memory effects appearing on this trace level analysis. The optimum pH value of the buffer mixed with plasma was found to be pH 9.0. Eksborg (18) mentioned pH 8.6 as an optimum, but he used an organic extraction solvent with a different composition.

As in several other investigations (9, 11, 13) the structural analogue daunorubicin was used as internal standard for comparable lipophilicity, and for comparable chemical and fluorescence properties. We found chloroform-1-heptanol (1:1) to be the best possible composition for the extraction of adriamycin, adriamycinol and daunorubicin (15). Using this organic solvent in a phase-volume ratio of 1:5, we achieved a 95% recovery of adriamycin for the whole clean-up procedure.

Eksborg (6, 17) determined the influence of the pH and the composition of the extraction mixture. His results indicate that an adriamycinol recovery of about 90% can be expected under these circumstances; this was confirmed in practice.

It is important to vortex the mixture of plasma and buffer for 45 sec since longer vibration yields in a smaller usable organic layer, because of a kind of emulsion of precipitated protein which is formed in the organic solvent. In order to achieve an almost quantitative transfer of adriamycin and adriamycinol to a small volume of the aqueous phase, the aqueous layer must be kept at an acidic pH.

By using 0.2 M H_3PO_4 in the aqueous phase, we obtained quantitative recovery and negligible aglycone formation. When aqueous, acidic solutions are stored for longer than about 3 h before analysis, it is preferable to use 0.1 M H_3PO_4, despite its lower yield. Also the life-time of the column will increase if the pH is increased.

Eksborg compared several chemically bonded phases for optimum separation of the drug, metabolite and plasma peaks and recommended the use of RP-8 bonded phases.

We investigated several commercially available RP-8 columns and selected the RP-8 column from Merck because of its very small plate height. The composition of the mobile phase (acetonitrile-water-0.1 M H_3PO_4, 37:60:3) was optimized for this column. For other types of columns the composition had to be changed for maximum separation. To prevent aglycone formation during the chromatographic process, the concentration of phosphoric acid should never exceed 0.1 M. The capacity factors (k') of adriamycin, adriamycinol and daunorubicin proved to be 1.7, 0.7 and 5.7, respectively, under these circumstances.

As in the sample treatment, the strong adsorptive properties of adriamycin interfere with the injection system and disturb the chromatographic processes and detection. Addition of desipramine-HCl diminishes this interference to a large extent.

In this procedure no gradient elution is needed; for routine analysis the flow-rate was kept at 2 ml min^{-1} to obtain short analysis times. Fig 1 shows a chromatogram of the analysis of a rat plasma sample. Calibration curves for standard solutions of adriamycin in aqueous solution were linear over the studied concentration range from 0.1 ng/100 µl to 10 µg/100 ul and passed through the origin (r^2 = 0.9999). Standard deviations in replication measurements were 1.5% in the higher range, and 3% in the lower range.

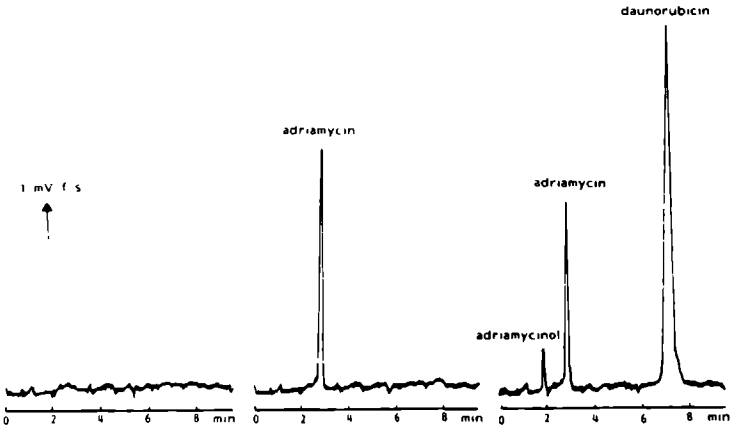


Fig. 1. A chromatogram of the analysis of rat plasma. Left, blank rat plasma; middle, blank rat plasma, spiked with 40 ng adriamycin/100 µl; right, plasma of treated rat, spiked with 60 ng daunorubicin/100 ul.

Calibration curves for the analysis of spiked plasma samples were linear over the studied concentration range of 1 ng/100 ul plasma up to 100 µg/100 µl plasma and passed through the origin (r^2 = 0.9999). Standard deviations in the analysis of plasma samples were 3% in the higher range, and 8% in the lower range.

PRELIMINARY PHARMACOKINETIC RESULTS IN RATS

We have developed the described assay of adriamycin in rat plasma in order to investigate the relation between the dosage, the method of administration of adriamycin and the toxic effects on heart tissue. To investigate the reliability of the method in pharmacological practice, we established the plasma concentration-time curves for several rats, on the basis of very small volumes of rat plasma The blood samples were collected according to the procedure described in the experimental chapter, namely by cannulating the vena jugularis. If the rats "disconnected" the cannule, blood samples were collected by orbital puncture.

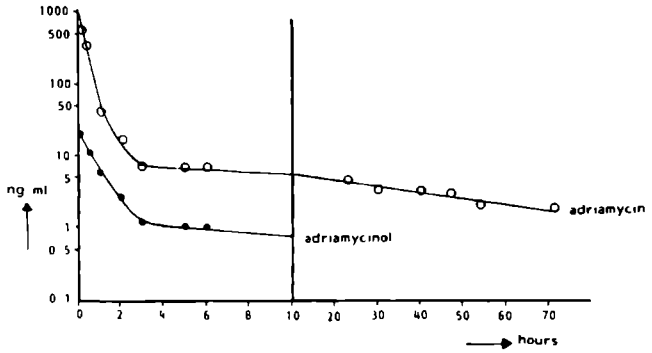


Fig. 2. A typical log plasma concentration-time curve for one rat (adriamycinol could only be analysed for up to 10 h).

Table 1.

TYPICAL PHARMACOKINETIC DATA FOR THE ANALYSIS OF ADRIAMYCIN OF THE log PLASMA CONCENTRATION-TIME CURVE FROM FIG 2

Wistar rat, 267 g, 2 mg adriamycin/kg by i v bolus injection

$C = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} + P \cdot e^{-\gamma t}$

A	996 6 ng ml^{-1}	α	5 17 h^{-1}	$t_{\frac{1}{2}\gamma}$	= 34 3 h
B	99 6 ng ml^{-1}	β	1 12 h^{-1}	V_{γ}	= 149 1 kg^{-1}
P	7 8 ng ml^{-1}	γ	0 020 h^{-1}	Cl_{tot}	= 0 050 1 min^{-1} kg^{-1}

A typical plasma concentration-time curve for adriamycin and adriamycinol is shown in Fig. 2. The line, shown in this figure, is the best fit, as calculated by the computer program. The pharmacokinetic data, calculated using this computer program, are collected in Table I. A three-compartment model gave the best fit. This highly sensitive method makes it possible to measure 6-8 half-lives. The pharmacokinetic data found in this way give new insight into the distribution and elimination of the drug. The distribution volume (V_γ), the half-life time of the elimination phase ($t_{1/2\,\gamma}$) and the total body clearance (CL_{tot}) are remarkably large, compared to earlier published data (19, 20). Further pharmacological experiments are in progress.

It can be concluded that the method described in this paper is reliable, sensitive and easy to use in pharmacological practice and may give us further insight into the pharmacology of adriamycin and its active metabolite in the rat and other species.

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Chapter 5

ANTITUMOR EFFECT, CARDIOTOXICITY AND NEPHROTOXICITY OF DOXORUBICIN IN THE IgM SOLID IMMUNOCYTOMA BEARING Lou/M Wsl RAT.

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ABSTRACT

Antitumor activity, cardiotoxicity and nephrotoxicity induced by doxorubicin were studied in LOU/M Wsl rats bearing a transplantable solid IgM immunocytoma. Animals with a tumor (diameter 15.8 ± 3.3 mm) were treated with i.v. injections of doxorubicin on five consecutive days, followed by one weekly injection for 7 weeks (dosage range from 0.015 - 4.0 mg/kg body weight). Tumor regression was observed with 0.5 mg/kg. Complete disappearance of the tumor was induced with 1.0 mg/kg. Histologic evidence of cardiotoxicity scored as grade III was only observed at a dose of 1.0 mg/kg. Light microscopic evidence of renal damage was seen above a dose of 0.5 mg/kg, which resulted in albuminuria and very low serum albumin levels. In the 1.0 mg/kg group the serum albumin level decreased from 33.6 ± 4.1 g/l to 1.5 ± 0.5 g/l. Ascites and hydrothorax were observed simultaneously. The same experiments were carried out with non-tumor-bearing rats, in which no major differences were observed.

In conclusion, antitumor activity, cardiotoxicity and nephrotoxicity were studied simultaneously in one and the same LOU/M Wsl rat. Albuminuria due to renal damage led to extremely low serum albumin levels. So ascites and hydrothorax are not necessarily a consequence of the observed cardiomyopathy, but may be due to low plasma osmotic pressure.

Doxorubicin has a well established place in the treatment of breast carcinoma, malignant lymphoma, acute lymphocytic and myelocytic leukemia, soft tissue sarcomas, and various other malignancies (1). Its clinical use is limited by cardiotoxicity. The incidence of cardiotoxicity sharply rises after a cumulative dose of 550 mg/m2 (2).

The cardiotoxicity of doxorubicin has urged for extensive research in order to improve the therapeutic index of this broad-spectrum antineoplastic agent. To study the cardiotoxicity, animal models have been described for the rabbit (3, 4, 5, 6, 7), the dog (8), the golden hamster (9) the rat (10, 11, 12) and the mouse (13, 14, 15). Any proposal for increasing the therapeutic index, whether it concerns the introduction of analogs, the prevention of deleterious oxidative processes or avoiding peak levels by altering the usual schedules, compels the investigator to show that the antitumor activity has not been lost. Except the Moloney-sarcoma virus induced sarcoma (16, 17) in the rat, we are not aware of a rat model in which the influence of any modification on both toxicity and antineoplastic effect on a solid tumor in the very same animal was studied simultaneously.

We decided to study the feasibility of the LOU/M Wsl rat bearing a solid immunocytoma as a model for doxorubicin-induced cardiotoxicity studies, allowing simultaneous studies of antitumor effects. As nephrotoxicity does occur in doxorubicin treated animals, special attention was also given to function and morphology of the kidney.

Animals

Breeding pairs of LOU/M Wsl rats and the IgM immunocytoma of LOU/C Wsl origin were kindly provided by Dr.H.Bazin (Catholic University of Louvain, Brussels, Belgium) (18). Animals were bred at our Institute. Male rats, weighing 170-220 g and 12 weeks of age were used, 8 animals per group in tumor-bearing experiments and 4-5 in the non-tumor-bearing experiments.

Housing conditions

The animals were housed in pairs in wire cages. Water and commercially available food (Muracon, Hope Farms, Woerden, The Netherlands) were provided ad libitum. The concentration of Vitamin E in the food was 67.5 mg/kg and selenium was present in trace amounts. Animal rooms were kept at a temperature of 22-25^{o}C and a relative humidity of 50-55%.

Tumor model

In the LOU/C inbred rat strain a high incidence of immunocytomas occurs secreting a variety of monoclonal immunoglobulins (19, 20). Because of poor breeding of the LOU/C rat in which the IgM tumor originally appeared, the histocompatible LOU/M rat was used as recipient for the IgM immunocytomas. Tumor cells, harvested by trypsinization (0.25% trypsin) of solid tumors, were stored in liquid nitrogen. The tumor cells in each experiment were used after one s.c. in vivo passage. The animals were inoculated subcutaneously on the left flank with 1 x 10^4 IgM immunocytoma cells in 0.5 ml plain RPMI-1640 medium (Gibco, Europe B.V., Hoofddorp, The Netherlands). The growth of the tumor was measured twice a week with a vernier callipers and expressed as mean value of three perpendicular measurements. Most animals injected with 1 x 10^4 IgM immunocytoma cells developed a palpable tumor after 17-21 days, which grew to a diameter of 2-3 cm within 6-7 days. At this time the tumor has metastasized to the regional lymph nodes and micrometastases in the liver can be detected.

Experimental design

Doxorubicin (Adriablastine^{R}, Farmitalia Carlo Erba Benelux, Brussels, Belgium) was prepared from commercially available 10 mg vials by reconstituting the lyophilized powder with sterile water to a concentration

of 2.0 mg/ml. For the lower concentrations the solution was diluted with physiological saline.

Two experiments were performed. The first experiment was done with tumor-bearing animals as described above. Eighteen days after tumor cell inoculation i.v. injections were started and performed on five consecutive days and then weekly. In this experiment the following dosages were used: no treatment; 0.015 mg/kg; 0.030 mg/kg; 0.060 mg/kg; 0.125 mg/kg; 0.250 mg/kg; 0.50 mg/kg; 1.0 mg/kg; and 2.0 mg/kg. The no-treatment group was injected with physiological saline (1.0 ml/kg body weight). Because the tumor secretes paraproteins, interference with protein metabolism and kidney function is to be expected. Therefore a second experiment was done without inoculation of tumor cells. In this experiment the same treatment schedule was applied and the following dosages were used: no treatment; 0.125 mg/kg; 0.250 mg/kg; 0.5 mg/kg; 1.0 mg/kg; 2.0 mg/kg; and 4.0 mg/kg. The first day of injection was assigned day 0. The animals were weighed once weekly. Blood was obtained once weekly by orbital puncture. Sera were stored at -20°C, for determination of total protein, albumin, and creatinine values. Proteinuria was assessed twice weekly. Tumor measurements were also done twice weekly. For histologic examination animals were killed under diethylether anaesthesia.

Histology

Tissue of the heart, liver, spleen, kidneys, lung, and the tumor inoculation site was fixed in 4% buffered formaldehyde and embedded in glycolmethacrylate or paraffin. One um glycolmethacrylate sections were made of the heart and the kidney. Longitudinal sections were made of the heart, which included the free wall of the right ventricle, the myocardial septum, the free wall of the left ventricle and the atria, and longitudinal and transverse sections were made of the kidneys. All 1 µm glycolmethacrylate sections were stained with hematoxylin-eosin like staining, Giemsa, toluidin blue, or periodic acid silver methenamine (PASM) and were examined with a light microscope. The lesions in heart tissue were scored according to Billingham (21). In the heart the number of mast cells was scored semi-quantitatively. Five um paraffine sections of liver, spleen and tumor inoculation site were stained with hematoxylin-eosin (HE).

Electron microscopy

To evaluate the light microscopy findings separately 4 control animals and animals treated with doxorubicin were studied after a cumulative dose of 12 mg/kg. At autopsy tissue of the heart was fixed by immersion in 2% glutaraldehyde in 0.1 M phosphate buffer. After postfixation in 1% Millonig's phosphate buffered OsO_4 and dehydration, the heart tissue was embedded in epon. One µm sections were stained with toluidin blue and studied with a light microscope. Ultrathin sections were cut with an LKB ultramicrotome (LKB, Bromma, Sweden), double stained with uranyl acetate and lead citrate and examined with a Philips EM 201 or 400 electron microscope.

Biochemistry

Proteinuria was assessed with Albustix^R (Ames, Division of Miles Nederland, Weesp, The Netherlands). The strongly positive samples were confirmed by gel electrophoresis.

Total protein was determined according to the method described by Lowry in an automatic device (Cabas Bio, Roche, Basel, Switzerland) simultaneously with the creatinine value, which was determined with a picrate method. The ratio albumin:total protein was obtained by analysing the extinction curve after gel electrophoresis of the sera. The areas under the curve were measured with electronic equipment (Videoplan, Kontron GmbH, Munich, GFR). The samples which showed very low albumin levels were analysed with electro-immunodiffusion according to Laurell, using rabbit anti-rat albumin serum for confirmation of the results (courtesy of Dr.A.M.Hagenaars, Department of Immunochemistry. RIVM).

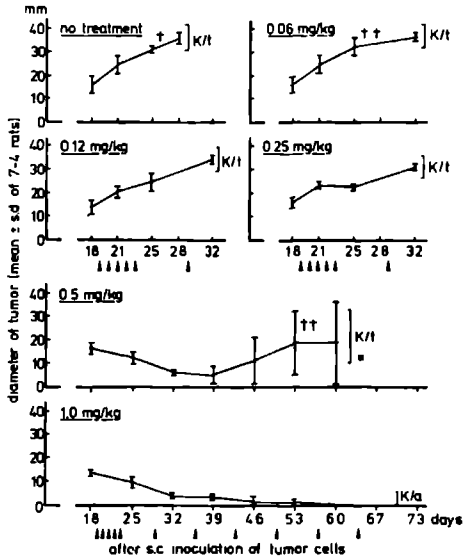
Statistics

Differences in group means were analysed by Student's t-test (two-tailed).

Clinical observations in tumor-bearing animals.

Antitumor effect; dose response relationship.

At the start of administration of doxorubicin (day 18), the diameter of tumors was 15.8 ± 3.8 mm. As shown in text-figure 1, in the no-treatment group the diameter on day 28 was 37.2 ± 3.2 mm. As death was suspected soon, these animals were killed. The 0.015 mg/kg and 0.03 mg/kg groups showed no inhibition of tumorgrowth and were killed on day 28 (data not shown). The tumors of the 0.06 mg/kg and 0.12 mg/kg groups reached a diameter of 37.2 ± 2.3 mm and 36.9 ± 3.1 mm on day 32; the animals of these groups were killed because of the tumor size. Shortly after the start of therapy the tumors of the 0.25 mg/kg groups showed inhibition of growth.



↓ = administration of drug † = 1 animal died
K/t = killed because of tumor size K/a = killed because of ascites
= 2 animals (without tumor) still alive at day 175

Text-figure 1. Effect of doxorubicin (iv administered) on growth of solid IgM immunocytoma in LOU/M rats.

However, after day 25 tumor growth was observed, and on day 32 the diameter of the tumor was 32.6 ± 1.7 mm. It was decided to kill these animals. Tumor regression was observed in the 0.5 mg/kg group. On day 39 the nadir of tumor diameter was reached: 5 mm. After day 39 tumor growth resumed except in two animals, which were still alive without tumor on day 175. Two animals died on day 53. The remaining animals were killed on day 73. A complete regression was obtained in the 1.0 mg/kg group and no recurrences were observed, however the animals were killed because of ascites on day 70. On day 32 the animals of the 2.0 mg/kg group had a palpable mass of 7.6 ± 5.1 mm (data not shown). These animals were killed on day 35 because of ascites.

Body weight, ascites and hydrothorax

The no-treatment group showed a slight increase in body weight during the observation period. Weight loss was seen in a dose-dependent way in the 0.5 mg/kg, 1.0 mg/kg, and 2.0 mg/kg group. The latter group showed during the remission-induction a loss of weight of 15%, followed by a gain which was related to clinically detectable ascites. For this reason the animals were killed. Ascites was also observed on day 52 after start of therapy in the 1.0 mg/kg group, for which reason they were killed. At autopsy ascites was found to be associated with hydrothorax.

Clinical observations in non-tumor-bearing animals.

Body weight, ascites and hydrothorax.

Administration of doxorubicin above 0.5 mg/kg resulted in weight loss, which was dose dependent. In the 1.0 mg/kg group weight loss of 15% was observed on day 42. Thereafter some animals developed ascites and hydrothorax, which was associated with weight gain. Animals of the 2.0 mg/kg group showed a reduction of body weight of 24% at day 7. These animals did not develop ascites. Ascites was neither observed in the 4.0 mg/kg group, which was killed already before day 7 because of extreme emaciation.

Histology

Heart. All animals of each group were studied, provided that fresh tissues were obtained. The number of animals examined is shown in parentheses in Table 1.

Table 1.
Semiquantitative assessment of 1 μm 2-hydroxyethyl methacrylate-embedded sections of morphologic changes in the myocardium in LOU/M rats after iv administration of doxorubicin.

Dose mg/kg	Cumulative dose, mg/kg	Day of assessment	n[a]	Cardiomyopathy grade[b]
		Tumor-bearing rats		
0		85	4	0
0.5	5.0	85	4	0
1.0	11.0	52, 53, 80	3	III
2.0	12.0	8	5	I
4.0	20.0	8	5	0
		Non-tumor-bearing rats		
0		10	7	0
0.125	1.75	14	2	0
0.250	3.5	14	7	0
0.5	7.0	42	3	0
1.0	14.0	56	6	III
2.0	10.0	14	6	I

[a] Number of animals assessed.

[b] Grading system according to Billingham et al. (21).

The lower dosage groups of both tumor-bearing and non-tumor-bearing animals showed no evidence of cardiac damage. In the 0.5 mg/kg groups no clear-cut morphologic changes were seen. In the 1.0 mg/kg groups however severe lesions were found: diffuse vacuolisation and extensive lysis of myofibrils (figs. 1-2) in both ventricular walls, without an area of predilection. They were classified as grade III cardiomyopathy. In the 2.0 mg/kg groups only sporadic lesions were seen, which were scored as grade I cardiomyopathy. In the 4.0 mg/kg group of non-tumor-bearing rats, which

were killed before day 7 no cardiac lesions were observed. There were no differences between tumor-bearing and non-tumor-bearing animals.

Semiquantitative evaluation of mast cell numbers did not yield a difference between the no-treatment groups and the doxorubicin-treated groups.

Ultrastructural investigation of damaged myocardium revealed distension of the sarcoplasmatic reticulum and the transverse tubular system, dissolution of the myofibrils with loss of the characteristic bands. In addition, disruption of the intercellular tight junctions was seen, suggesting that the loss of mechanic coupling of the cells augments the detrimental effects of doxorubicin. The figures 3 and 4 are representative for the observed myocardial lesions.

Kidney. Examination of the kidneys revealed no histologic changes in the no-treatment groups of both tumor-bearing and non-tumor-bearing animals (Figs. 5-6). Above 0.25 mg/kg lesions became evident. Marked alterations in the periphery of the glomeruli were seen, with synechial bands between the parietal and visceral leaves of Bowman's capsule, actually obliterating its space (Fig. 7). In the tubuli protein casts were seen (Fig. 8). The epithelium showed degenerative and regenerative changes with polymorphism of nuclei and numerous mitotic figures. In the epithelium of the proximal tubules fine granules were seen, which in additional histochemical staining showed resemblance to haemosiderin. No calcifications were seen.

Liver and spleen. In the no-treatment group of tumor-bearing animals the liver was displaced by tumor cells. Microscopically the liver was only recognizable as such by the characteristic distribution of portal areas and central veins. With increasing dosages of doxorubicin less tumor cells were seen in the liver. In the 1.0 mg/kg and the 2.0 mg/kg groups no metastases in the liver were seen. Similar results were obtained with the spleen.

Tumor inoculation site. In the 1.0 mg/kg treatment groups no viable tumor cells were seen in the tumor inoculation site. However a massive accumulation of pigment-laden macrophages were found containing brown and yellow granules. By additional Perl's staining the former could be identified as hemosiderin. In addition, the macrophages showed yellow autofluorescence, identifying the second pigment as ceroid.

Fig. 1. Cross section of myocardium of a tumor-bearing rat treated with 1 mg/kg doxorubicin, showing diffuse vacuolisation of myocardial fibers.
1 μm 2-hydroxyethyl methacrylate embedded section; Giemsa staining; 320x.

Fig. 2. Longitudinal section of myocardial fibers of a tumor-bearing rat treated with 1 mg/kg doxorubicin showing lysis and vacuolisation.
1 μm 2-hydroxyethyl methacrylate embedded section; Giemsa staining; 320x.

Fig. 3. Extensive vacuolization of the sarcoplasmic reticulum of a cardiac myofiber from a doxorubicin treated tumor-bearing rat. The appearance of all mitochondria is normal; 6500x.

Fig. 4. Electron micrograph of a doxorubicin treated tumor-bearing rat. At the left side of the intercalated disk all myofibrils are widely separated and disintegrated. The Z-bands are out of register; 9500x.

Fig. 5. Picture of renal cortex of a tumor-bearing rat of the no treatment group showing quite regular basement membrane of the glomerular tufts, intact brush border in the proximal tubules and regular nuclei.
1 μm 2-hydroxyethyl methacrylate embedded section; PASM staining; 320x.

Fig. 6. Detail of tubular structures in the renal cortex of a tumor bearing rat of the no treatment group showing regular nuclei and an intact brush border.
1 μm 2-hydroxyethyl methacrylate embedded section; Giemsa staining; 320x.

Fig. 7. Renal cortex of tumor-bearing rat treated with 1 mg/kg doxorubicin. There is proliferation of cells of the parietal leave of Bowman's capsule (arrow). Bands seem to have been formed between the parietal and visceral leaves of Bowman's capsule.
1 μm 2-hydroxyethyl methacrylate embedded section; PASM staining; 320x.

Fig. 8. Detail of the renal cortex of a tumor-bearing rat of the 1 mg/kg

group. The proximal tubuli are composed of cubic cells, which lost their brush border. The nuclei show a marked polymorphism. Note the presence of protein casts.
1 µm 2-hydroxyethyl methacrylate embedded section; Giemsa staining; 320x.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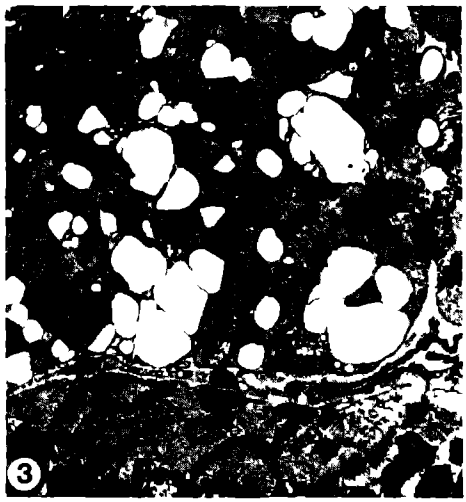
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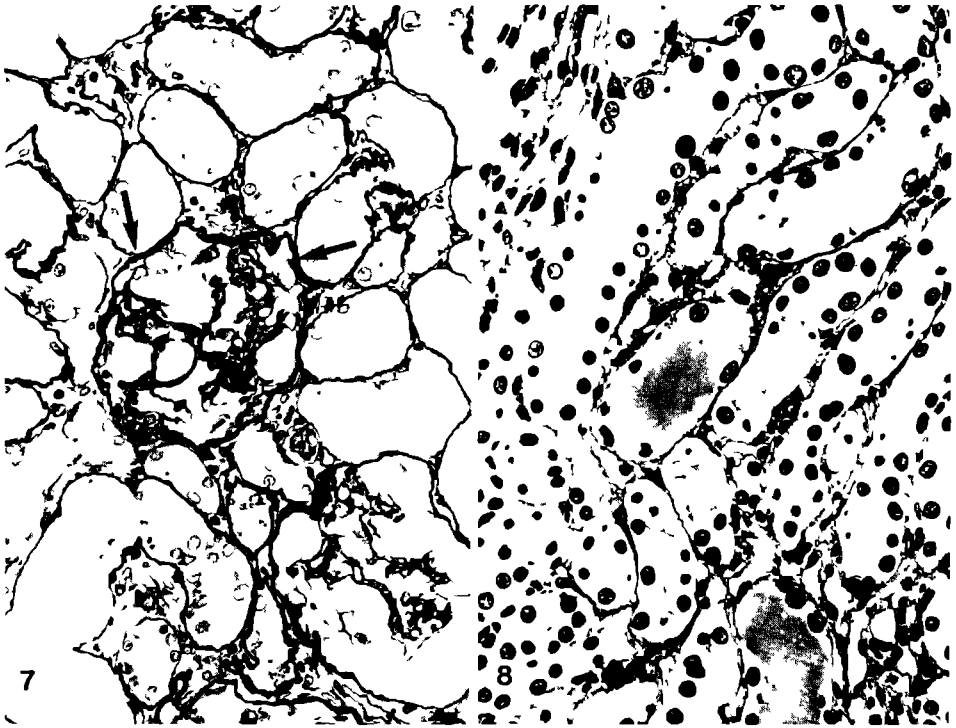
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Table 2.

Serum albumin levels in LOU/M rats during administration of doxorubicin

Doxorubicin dose, mg/kg	Serum albumin levels[a] at days after start of doxorubicin treatment				
	0	7	14	42	56
			Tumor-bearing rats		
0.5	31.6 ± 2.1 (8)	29.8 ± 1.9 (8)	31.0 ± 3.4 (8)	23.7 ± 7.1 (5)	25.4 ± 2.9 (3)
1.0	33.6 ± 4.1 (8)	28.9 ± 2.1 (8)	27.3 ± 2.6 (8)	6.2 ± 2.4 (7)	1.5 ± 0.5 (8)
2.0	24.9 ± 10.3 (7)	26.9 ± 4.4 (8)	4.0 ± 1.3 (8)	-	-
			Non-tumor-bearing rats		
0.5	40.0 ± 8.5 (2)	40.6 ± 1.8 (2)	32.5 ± 1.9 (4)	23.9 ± 4.5 (4)	22.4 ± 3.9 (4)
1.0	41.3 ± 0.5 (2)	38.1 ± 2.1 (3)	21.6 ± 2.3 (4)	9.9 ± 8.0 (3)	-
2.0	44.0 ± 3.6 (5)	22.6 ± 3.0 (3)	-	-	-

[a] Mean and SD of albumin levels are expressed in g/liter.
Numbers in parentheses are numbers of observations.

[b] $P < 0.05$ (vs. day 0).

[c] $P < 0.01$ (vs. day 0).

[d] $P < 0.001$ (vs. day 0).

Serum albumin concentration. On day 0 the mean serum albumin concentration of all animals used in the tumor-bearing experiment was 31.2 ± 4.4 g/l and in the non-tumor-bearing experiment it was 40.8 ± 4.9 g/l. This difference was statistically significant ($p < 0.001$; Student's t-test, two-tailed).

Tumor-bearing animals. Seven days after starting therapy in the no treatment group of tumor-bearing animals the serum albumin level decreased to 21.9 ± 3.1 g/l, probably due to the loss of liver parenchyma, resulting in impaired albumin production . With increasing doses of doxorubicin the decrease of the serum albumin level was less pronounced but was statistically significant for all lower dosage groups (data not shown).

Table 3.
Assessment of urinary albumin concentrations in LOU/M rats during iv administration of doxorubicin

Doxorubicin dose, mg/kg	Urinary albumin concentration at days after start of doxorubicin treatment[a]				
	0	7	14	42	85
	Tumor-bearing rats				
0	+	++	++		
0.125	+	++	++		
0.250	+	++	++		
0.5	+	+	+	+++	
1.0	+	++	+++	++++	
2.0	+	+++	++++		
	Non-tumor-bearing rats				
0	+	+	+	+	+
0.125	+	+	+	+	+
0.250	+	+	+	++	+++
0.5	+	+	+	++	+++
1.0	+	+	++	+++	++++
2.0	+	++			
4.0	+	+++			

[a] Summary of the weekly assessments: + = trace amount, + = 0.3-1.0 g/liter, ++ = 1.0-3.0 g/liter, +++ = 3.0-10.0 g/liter, ++++ => 10.0 g/liter.

In the 0.5 mg/kg group which obtained a temporary tumor remission a fairly constant albumin concentration was seen. Serum albumin levels of rats treated with 1.0 and 2.0 mg/kg declined sharply to near zero levels (table 2). These results were confirmed with the electroimmunodiffusion assay according to Laurell with rabbit anti-rat albumin sera.

Non-tumor-bearing animals. From day 42 the 0.5 mg/kg group showed a serum albumin level, which was significantly lower than the prior treatment level ($p < 0.05$) (table 2). The 1.0 mg/kg group showed from day 14 a significant decrease in serum albumin level ($p < 0.01$) and at day 42 a very low serum albumin level of 9.9 ± 8.0 g/l. The 2.0 mg/kg group showed already on day 7 a significant decrease in albumin level ($p < 0.001$).

Albuminuria

At day 0 the albumin concentration in urine in both the tumor-bearing and non-tumor-bearing animals was between trace amount and 1.0 g/l (table 3) animals. A dose dependent increase of proteinuria was observed, reaching levels > 10 g/l for the 1.0 mg/kg and 2.0 mg/kg respectively on day 14 and 42. In non-tumor-bearing animals this level was found in the 1.0 mg/kg group on day 85.

Serum creatinine levels

Prior to the administration of doxorubicin, the serum creatinine level of tumor-bearing rats was higher than the serum creatinine level of non-tumor-bearing rats (67.8 ± 5.6 vs. 51.8 ± 2.6) (Table 4). During the treatment of the tumor-bearing animals a gradual decrease of serum creatinine level was noticed already after 7 days. A dose dependent effect was seen on day 14 above a dose of 0.125 mg/kg body weight doxorubicin. On day 49 the 0.5 and 1.0 mg/kg groups had obtained a serum creatinine level comparable to the pretreatment level of non-tumor-bearing animals (respectively 50.9 ± 1.6 and 52.6 ± 10.7). At that time the remaining animals were free of tumor except one in the 0.5 mg/kg group.

The non-tumor-bearing animals showed during the experiment some fluctuation in serum creatinine level. At the end-point of the experiment however, no significant change was observed.

Table 4.

Serum creatinine levels in LOU/M rats during administration of doxorubicin

Doxorubicin dose, mg/kg	Serum creatinine levels at days after start of doxorubicin treatment				
	0	7	14	42	35
	tumor-bearing rats				
0.5	69.7 ± 5.5 (8)	62.6 ± 2.8 (8)	70.0 ± 5.5 (8)	55.8 ± 3.7 (5)	50.9 ± 1.6 (3)
1.0	73.4 ± 5.2 (8)	61.0 ± 3.1 (8)	60.3 ± 3.1 (8)	39.7 ± 7.2 (6)	52.6 ± 10.7 (8)
2.0	67.3 ± 4.1 (7)	56.4 ± 4.7 (8)	52.0 ± 3.7 (6)	-	-
	Non-tumor-bearing rats				
0.5	46.6 ± 1.4 (2)	54.5 ± 1.6 (2)	51.9 ± 1.9 (4)	63.9 ± 2.1 (4)	58.9 ± 6.3 (4)
1.0	53.6 ± 3.2 (2)	58.3 ± 1.9 (3)	50.5 ± 4.5 (4)	56.7 ± 3.9 (3)	46.8
2.0	50.0 ± 2.0 (5)	75.4 ± 5.8 (3)	-	-	-

[a] Mean and SD of creatinine levels are expressed in μmol/liter. Numbers in parentheses are numbers of observations.

[b] $P < 0.01$ (vs. day 0).

[c] $P < 0.001$ (vs. day 0).

[d] $P < 0.05$ (vs. day 0).

This study has shown that the IgM immunocytoma in the LOU/M Wsl rat is susceptible for doxorubicin in a dose dependent way. The 1.0 mg/kg group treated with the schedule described in the section Materials and Methods, showed a complete regresion of tumor without recurrences. The lower dosage groups (0.015 mg/kg - 0.5 mg/kg doxorubicin) experienced antitumor activity, which was insufficient, i.e. death in these treatment groups was due to tumor growth. The 2.0 mg/kg dose in both tumor-bearing and non-tumor-bearing animals, and the 4.0 mg/kg dose in non-tumor-bearing animals caused early death due to acute toxicity, not related to cardiomyopathy.

The well-known pathology of anthracycline induced cardiomyopathy was reproduced in the 1.0 mg/kg and 2.0 mg/kg groups. When light microscopy findings were compared to electron microscopy observations, it is concluded that the 1 μm light microscopy technique was adequate for assessing cardiomyopathy.

The 2.0 mg/kg group received a cumulative dose, which was approximately equal to the cumulative dose in the 1.0 mg/kg group (12.0 versus 11.0 mg/kg), suggesting that development of cardiomyopathy is not only dose dependent but also time dependent. The 1.0 mg/kg dose represents a compromise dose between the dose needed for antitumor activity and a dose at which acute toxicity is non-lethal, permitting the anthracycline related cardiomyopathy coming to expression.

Besides the cardiomyopathy, extensive renal damage could be demonstrated, leading to proteinuria and extreme hypoalbuminaemia in a dose-dependent way, both in tumor-bearing and non-tumor-bearing animals. The lower serum albumin levels in the tumor-bearing animals could be accounted for by the combination of diminished synthesis due to metastatic liver disease in addition to the high protein demand of the immunocytoma. Furthermore urinary loss of paraproteins could have contributed to this effect. Extremely low serum albumin levels were observed.

The higher serum creatinine levels in tumor-bearing animals in comparison to non-tumor-bearing animals may be explained by the detrimental effects of tumor products on creatinine clearance. The treatment with doxorubicin resulted in parallel with tumor load reduction in an improvement of serum creatinine values. The physiological meaning of the

minor changes observed is however doubted. In the non-tumor-bearing experiment no trend in serum creatinine values could be observed: the proteinuria evoked by doxorubicin is not accompanied by an increase of serum creatinine values.

Nephropathy in the rat in relation to treatment with an anthracycline has been mentioned early in literature (22). Accumulation of body fluids has been accepted as a manifestation of chronic heart failure due to anthracycline induced cardiomyopathy (4, 23, 24). Also in our study development of ascites was observed in the 1.0 mg and 2.0 mg/kg group of tumor-bearing animals, which correlated with a rapid increase of body weight.

However, not only heart failure, but also loss of plasma oncotic pressure due to hypoalbuminemia may cause accumulation of fluid in the body. Hypoalbuminemia has been mentioned in literature (25, 26, 27). Detailed studies on serum albumin levels were reported by Bristow (26) for the rabbit and by Bertani (25) for the rat. In neither study the near zero level which we observed was reported. The extremely low albumin levels could be the results of strain-related different sensitivity for the anthracycline induced effects, for the autoanalyzer method used by Bristow (26) and the gelelectrophoresis used by Bertani (25) gave comparable results. Bertani studied serum albumin levels after a single administration of doxorubicin 7.5 mg/kg, whereas this study describes serum albumin levels after repeated administration of low doses doxorubicin. The hypocalciaemia observed by Mettler could be related to hypoalbuminaemia (23).

The loss of albumin and therewith the loss of plasma colloid osmotic pressure should have tremendous consequences for the circulation and may be responsible for ascites and hydrothorax. In literature on the human nephrotic syndrome a distinction is made between a high-renin, high-aldosterone with vasoconstriction variant and a low-renin, low-aldosterone with sodium-overloading variant (28). Pilot-studies in our Institute have shown that after treatment with doxorubicin <u>in vivo</u> a decreased heart-minute-volume could be demonstrated, whereas the same hearts showed no signs of cardiac failure by the method of Langendorff (unpublished data), suggesting that contractility is intact and vasoconstriction plays a role in these <u>in vivo</u> results. In addition to the consequences of hypoalbuminaemia for the circulation the role of anthracycline-induced vasoactive substances recently have been emphasized (26, 29). In our study

no influence of doxorubicin on the number of mast cells in the heart was observed.

In conclusion, since the search for less cardiotoxic anthracycline analoga or variations in posology with retaining antitumor activity is still relevant for human medicine, animal models are needed for testing the effects of changes in treatment. Besides the Moloney Sarcoma Virus induced osteosarcoma we are not aware of a rat model, which allows the simultaneous assessment of antitumor activity and cardiotoxicity (17, 24). However, interpretation of data on cardiomyopathy in animal models should be accompanied by data on nephrotoxicity, since gross findings of ascites and/or hydrothorax are not only explained by heart failure but also by hypoalbuminemia, which is observed in anthracycline treated rats and rabbits.

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Chapter 6

Time course study on doxorubicin induced nephropathy and cardiomyopathy in male and female LOU/M Wsl rats.

Lack of evidence for a causal relationship.

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SUMMARY

In a previous study on doxorubicin induced cardiotoxicity in LOU/M Wsl rats severe nephropathy has been observed. Therefore, the question was raised whether nephropathy adds to, or even might be responsible for doxorubicin induced cardiomyopathy in rats. To elucidate this question we studied the temporal relationship between the onset of doxorubicin induced cardiomyopathy and nephropathy. In addition, we examined whether modifications of the treatment schedule could circumvent nephrotoxicity. Because preliminary studies had shown that female LOU/M Wsl rats developed less doxorubicin induced albuminuria, both male and female LOU/M Wsl rats were treated with doxorubicin 1 mg/kg dose i.v. on 5 consecutive days and then weekly. Saline treated animals served as controls. Albuminuria, serum albumin, and serum creatinine levels were assessed weekly. For histologic examination 5 male and 5 female rats were killed weekly.

At day 14 and thereafter male doxorubicin treated rats showed albuminuria > 10 g/l. Albuminuria of > 10 g/l was not avoided by modifications of the treatment schedule. Female rats had on day 14 an urinary albumin level of 1.0 - 3.0 g/l, yet reaching the > 10 g/l level at day 49. In male rats serum albumin levels decreased to levels below 10 g/l

($p < 0.001$ vs. day 0); in contrast female rats maintained serum albumin levels till day 49. Serum creatinine levels showed a tendency to rise, the values of male rats not being measured after day 28 due to hyperlipidemia, the levels of female rats increased from 37.8 ± 3.0 µmol/l to 53.7 ± 2.5 µmol/l on day 49 ($p < 0.001$). At day 10 a grade 1 - 1.5 cardiomyopathy score, assessed according to the modified Billingham scoring system, was found, both in males and females, gradually increasing to grade 2.5 - 3 cardiomyopathy on day 49. In male LOU/M rats the nephropathy developed steadily from day 14 and thereafter, whereas in females the rate of development of kidney damage was slower and at the endpoint of the study the severity of kidney lesions less in comparison to males. The onset of cardiomyopathy and nephropathy was simultaneously. It is concluded that cardiomyopathy observed in LOU/M Wsl rats is a phenomenon independent of nephropathy.

Rats and rabbits are frequently used to study the cardiomyopathy induced by doxorubicin and related compounds (1, 2). In both species the long term toxic effects of doxorubicin consist not only of cardiomyopathy, but also of nephropathy (1-4). Morphologically the renal damage is characterized by glomerular, tubular, and interstitial pathology (2-5). Functionally, the nephropathy in the rat causes albuminuria and hypoalbuminemia finally resulting in nephrotic syndrome resulting.

In clinical medicine doxorubicin induced nephropathy is not observed. One might conclude therefore, that doxorubicin cardiomyopathy and nephropathy are independent phenomena. Van Vleet et al., however, have concluded that doxorubicin induced nephropathy represents an important cause of death in chronically treated rabbits and detracts greatly from the value of the rabbit as a model for chronic cardiomyopathy studies (6).

The IgM immunocytoma in the LOU/M Wsl rat has shown to be a tumor system, in which toxicity and antitumor activity of doxorubicin and platina compounds have been studied simultaneously (1, 2, 7). In addition, the entrapment of doxorubicin into negatively charged liposomes has proven to decrease the toxic effects of doxorubicin on kidney and myocardium, with preservation of the antitumor effect (1). Functional studies in our Institute have provided clues for the activation of compensatory mechanisms for the negative effects of doxorubicin induced nephrotoxic syndrome (8). After a cumulative dose of 7 mg/kg body weight, non pre-instrumented anaesthetized LOU/M Wsl rats demonstrated on day 25 after start of administration of doxorubicin in vivo a higher myocardial performance when compared with control rats. In addition, the inotropic response towards dobutamine or norepinephrine as well as the vasoconstrictor response towards norepinephrine were profoundly impaired. However, the isolated and perfused rat heart showed on day 25 after the same treatment schedule with doxorubicin no changes in myocardial contraction under basal nor inotropic stimulatory conditions.

Considering the conclusion of Van Vleet about the relationship between nephrotoxicity and cardiomyopathy, and the aforementioned functional data obtained at our Institute, we decided to investigate whether the nephropathy in the LOU/M Wsl rat detracts from its value as a model for studies on doxorubicin induced cardiomyopathy.

Previous studies (1, 2) in our Institute had indicated that doxorubicin 1 mg/kg dose on five consecutive days and thereafter weekly provided a balance between desired antitumor activity and acute toxicity, ultimately resulting in grade 3 cardiomyopathy according to the Billingham score (9), severe hypalbuminemia, and severe nephropathy. In kidney tissue after one single dose of 1 mg/kg doxorubicin at 4 h after i.v. injection a doxorubicin tissue level of 12.97 ± 1.00 mg/kg and at 24 h a level of 5.58 ± 1.10 mg/kg have been found (1). The administration of drug on five consecutive days might result in drug accumulation in the kidney. Therefore, the influence of modifications in the dose schedule, eventually resulting in a cumulative dose of 10-13 mg/kg doxorubicin on day 70 after start of treatment, on nephrotoxicity assessed by albuminuria, has been investigated.

To elucidate the sequence of events, we have performed a time course study on the relationship in time between the occurrence of nephropathy and cardiomyopathy in the LOU M/Wsl rat. Preliminary studies had shown that after administration of doxorubicin male LOU M/Wsl rats developed albuminuria, and consequently hypalbuminemia, which were more severe and occurred earlier, than in female rats. Therefore, the severity of nephropathy and cardiomyopathy is compared weekly in both male and female LOU/M Wsl rats. A divergence in severity of nephropathy and cardiomyopathy in any sex would point at the independence of both phenomena in this animal system.

Animals. Breeding pairs of LOU/M Wsl were kindly provided by Dr.H.Bazin (Catholic University of Louvain, Brussels, Belgium). Animals were bred at the National Institute of Public Health and Environmental Hygiene. Female and male animals weighing 162 ± 12 g (n = 43) and 259 ± 21 g (n = 43) respectively, and 12 weeks old were used. The animals were housed in pairs in wire cages. Water and commercially available food (Muracon, Hope Farms, Woerden, The Netherlands) were provided ad libitum.

Experimental Design. Doxorubicin (Adriablastine^{R}, Roger Bellon S.A. Neuilly sur Seine, France) was obtained commercially. The lyophilized powder was reconstituted with sterile water to a concentration of 2.0 mg/ml. The solution was further diluted to a concentration of 1.0 mg/ml with saline 0.9% w/v. In the part of the study, which dealt with the effect of different treatment schedules, only male LOU/M Wsl rats were used. Four groups were formed, each consisting of 6 animals. Doxorubicin was administered into the tail vein at a 1 mg/kg dose. Group 1 was treated on days 1, 2, 3, 4, 5, 12, 19, 26, 33, 40, 47, 54 and 61, obtaining a cumulative dose of 13 mg/kg. Group 2 was treated on days 1, 4, 11, 18, 25, 32, 39, 46, 53, 60 and 67, cumulative dose 11 mg/kg. Group 3 was treated on days 1, 8, 15, 24, 31, 38, 45, 52, 59 and 66, cumulative dose 10 mg/kg. Group 4 was treated on days 1, 4, 15, 18, 29, 32, 43, 46, 57 and 59, cumulative dose 10 mg/kg. Urinary albumin concentration was assessed twice weekly.

In the time course study, doxorubicin was administered i.v. in a 1 mg/kg dose on five consecutive days and then weekly in male and female rats. In previous studies this regimen had shown both to have antitumor activity against an IgM immunocytoma and to induce damage to the structure of kidney and myocardium (2). This regimen did not result in weight loss as determined by weekly assessment. The last administration was on day 47. On days 0, 10, 14, 21, 28, 35, 42, 49, five rats of both sexes were killed. The histology of kidney and heart of doxorubicin treated animals was compared with that of saline treated control animals which were autopsied on day 49 after start of treatment.

For light microscopic evaluation tissue of the heart and kidney was fixed in 4% buffered formaldehyde and embedded in 2-hydroxyethyl methacrylate. One µm sections were made and stained with Giemsa.

Scoring system. Cardiac lesions were graded according the modified scoring system, proposed by Torti et al. (7) (Table 1). Kidney lesions were scored on a severity scale with the following gradings: no lesions (0); minor lesions (+); moderate lesions (++); and severe lesions (+++). The following items were assessed: alterations of glomerular basal membranes, changes in glomerular epithelial cells, the distribution of lesions through the glomerulus (segmental versus global), and the distribution of the lesions through the renal cortex (focal versus diffuse).

TABLE 1

Cardiomyopathy scoring system[1)]

Grade	Description
0	normal cells
1	< 5% of the cells have myofibrillar loss or distended cytoplasmic vacuolisation or both
1.5	5-15% of the cells have myofibrillar loss or cytoplasmic vacuolisation, or both
2.0	16-25% of the cells show changes as described above
2.5	26-35% of the cells show changes as described above
3.0	> 35% of the cells show changes as described above

1) Adapted from M.E.Billingham (8)

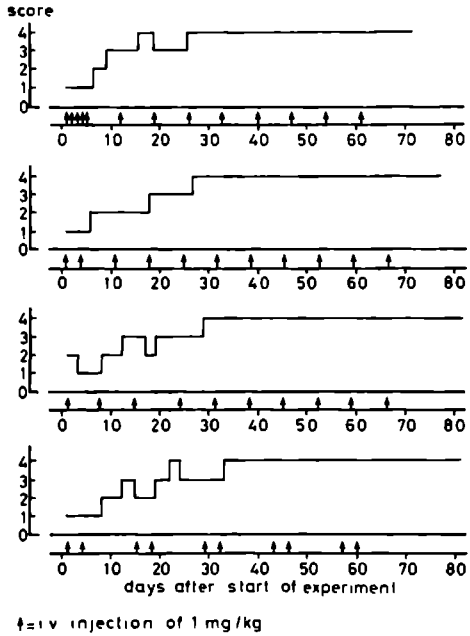
For electron microscopic evaluation, 1 mm^3 blocks of kidney tissue were fixed by immersion in 2% glutaraldehyde in 0.1 M cacodylate buffer. After postfixation in 1% cacodylate buffered OsO_4 and dehydration the kidney tissue was embedded in epon. One µm sections were stained with toluidine blue and studied with a light microscope. Electron microscopic work was done on selected sections. Selection was based on severity of lesions observed in light microscopic survey. Ultrathin sections were cut with an LKB ultramicrotome (LKB, Bromma, Sweden), double stained with uranyl acetate and lead citrate, and examined with a Philips EM 201 or 400 electron microscope.

Biochemistry. Blood was obtained weekly by orbital puncture and before autopsy by puncture of the aorta under ether anaesthesia. The serum albumin level was assessed by a brillant cresyl-purple method and serum creatinine levels with a picrate method in an automatic device (Cobas Bio, Roche, Basel, Switzerland). Urinary albumin concentrations were assessed weekly by Albustics^R (Ames, Division of Miles Nederland, Weesp, The Netherlands).

Statistics. Differences in group means were analysed by Student's t-test (two-sided). In case of insufficient homogeneity of variances, the Welch correction with respect to the degrees of freedom was applied.

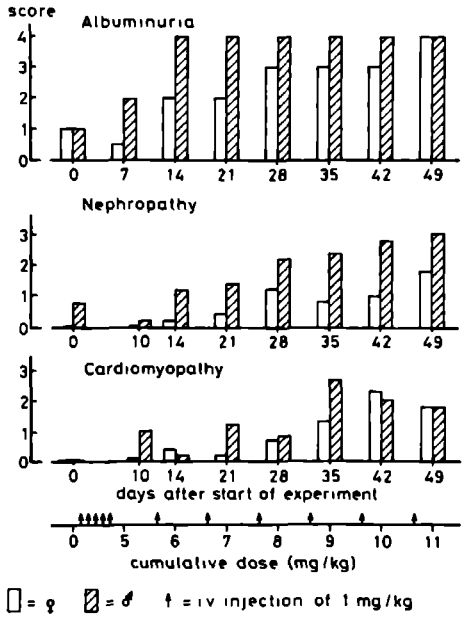
Albuminuria

In the part of the study on modifications of the treatment schedule, animals of group 1 reached an urinary albumin concentration of > 10 g/l on day 16 after start of treatment (Text-figure 1). On day 32, all animals in groups 1-4 had urinary albumin concentrations of > 10 g/l. This level of albuminuria correlated with a cumulative dose of doxorubicin varying from 4 to 6 mg/kg. This line of investigation has not been continued.



Text-figure 1: Albuminuria in Lou/M rats induced by doxorubicin in different treatment schedules.
urinary albumin concentration: 1 = 0.3 - 1.0 g/l; 2 = 1.0 - 3.0 g/l; 3 = 3.0 - 10.0 g/l; 4 = > 10.0 g/l.

Both female and male LOU/M Wsl rats have spontaneously albuminuria in the range 0.3 - 1.0 g/l (Text-figure 2). After administration of doxorubicin a rapid increase in urinary albumin concentration occurred. At day 14 male rats reached an urinary albumin concentration of > 10 g/l. Female rats, however, had at day 14 an urinary albumin concentration in the range of 1.0 - 3.0 g/l, increasing to the range of 3.0 - 10.0 g/l at day 28 and thereafter. Not before day 49 female rats obtain similar urinary albumin concentrations as their male counterparts.



Text-figure 2: Time relationship between albuminuria, nephropathy, and cardiomyopathy in Lou/M rats during treatment with doxorubicin.

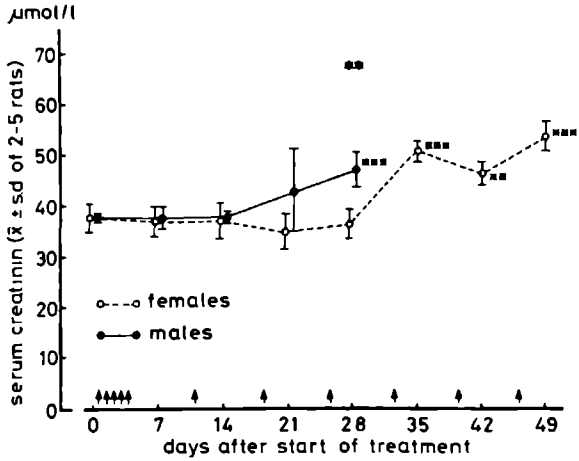
↑ = i.v. injection of 1 mg/kg

urinary albumin concentration: 1 = 0.3-1.0 g/l; 2 = 1.0-3.0 g/l; 3 = 3.0-10.0 g/l; 4 = >10.0 g/l.

Nephropathy: 0 = no lesions; 1 = minor lesions; 2 = moderate lesions; 3 = severe lesions.

Serum albumin levels

The serum albumin levels after prolonged doxorubicin treatment of LOU/M Wsl male and female rats are presented in Text-figure 3. In contrast to the female rats, male ones showed a rapid and strong decrease in serum albumin level, starting already at day 14 after the first DXR treatment (cumulative dose 6 mg/kg). From day 0 until day 49 the serum albumin levels of female LOU/M Wsl rats exhibited a slight tendency to decrease but did



Text-figure 3: Serum albumin levels in male and female Lou/M rats during i.v. treatment with doxorubicin.

↑ = i.v. injection of 1 mg/kg.

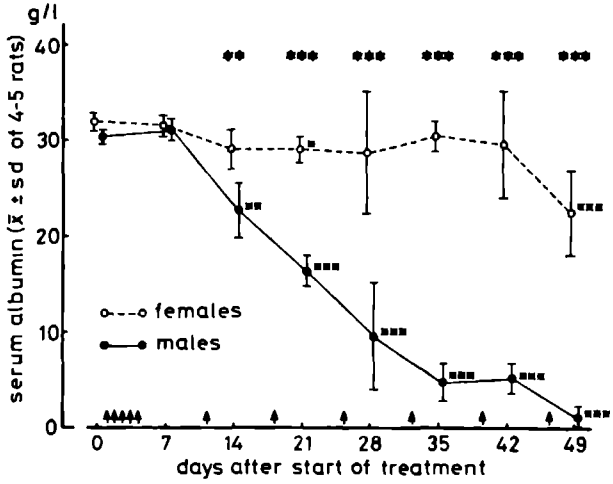
* : $p < 0.05$; ** : $p < 0.01$; *** : $p < 0.001$ compared to day 0;#$p < 0.05$ compared to female rats on the same day

$p < 0.01$; ### $p < 0.001$.

not differ significantly, except for levels on days 21 and 49. At the last day the difference reached the significance level of $p < 0.001$, the cumulative dose being 11 mg/kg. Meanwhile male LOU/M Wsl rats had serum albumin levels of almost zero, reflecting the severe albuminuria in these animals.

Serum creatinine levels

Serum creatinine levels of female LOU/M Wsl rats had increased significantly from a value of 37.8 ± 3.0 umol/l at day 0 to 50.6 ± 2.1 umol/l at day 35 and thereafter (Text-figure 4). Male LOU/M Wsl rats showed after day 14 a gradual increase of serum creatinine level which



Text-figure 4: Serum creatinine level in male and female Lou/M rats during i.v. treatment with doxorubicin. ↑ = i.v. injection of 1 mg/kg. ** : $p < 0.01$; *** : $p < 0.001$ compared to day 0; ✱✱: $p < 0.01$ compared to female rats on the same day.

however did not reach statistical significance. After day 28 serum creatinine levels in the males could not be measured any longer, due to turbidity of the hyperlipidemic serum.

Cardiomyopathy

Low cardiomyopathy scores were observed for male and female LOU/M Wsl rats till day 35. On the average the score of male rats was during this period of observation slightly ahead of the score of female ones. On day 35 and thereafter a sharp increase in cardiomyopathy score for both sexes was seen. No essential difference between male and female LOU/M Wsl rats could be ascertained after day 35. The cardiac lesions consisted of vacuolisation and loss of myofibrils. The lesions were in accordance with those described by us previously (1, 2). In control animals no cardiomyopathic changes were observed. A compilation of data is presented in Table 2. The mean cardiomyopathy score is shown in Text-figure 2.

TABLE 2

Cardiomyopathy score in male and female Lou/M Wsl rats by time and cumulative dose doxorubicin

Day	Cumulative dose mg/kg	grade						
		0	1	1.5	2	2.5	3	n[2)]
male								
0	0							
10	5	1	1	1	1	1		5
14	6	3	2					5
21	7			2	1			4
28	8		3	1	1			5
35	9					1	2	3
42	10			1	2		2	5
49	11			1	1		1	3
49	0	2						2
female								
0	0							
10	5	4	1					5
14	6	2	1	1				4
21	7	3	2					5
28	8		3	2				5
35	9			3	1	1		5
42	10			2			2	4
49	11				2	2		4
49	0	2						2

1) scoring system adapted from M.E.Billingham (8)

2) n = number of animals

Nephropathy

At light microscopic examination the early stage of doxorubicin induced nephropathy was characterized by thickening of the epithelial lining of the parietal leave of Bowman's capsule. Subsequently delicate adhesions between the parietal and visceral leave were noticed, simulating epithelial blebs. Continuation of the administration of doxorubicin resulted in progressive destruction of glomerular architecture. The end stage of doxorubicin induced glomerulopathy was characterized by shrinkage of the glomerular structure, with complete absence of glomerular tufts, where in only a few nuclei were seen. During the development of the eventual glomerulopathy no cellular infiltrate occurred. No predilection for any region of the renal cortex could be revealed. In the capillaries no thrombi were seen. Crescent formation was absent. Examples of various degrees of severity of glomerulopathy are shown in Figs. 1-5.

Very soon after the start of administration of doxorubicin protein casts appeared. The diameter of granules in cells of the proximal tubuli increased during the early stage of kidney damage. Later on, the brush border disappeared, and simultaneously the granules were no longer detectable. Eventually, atrophy of tubuli occurred. No conspicuous lesions in the interstitium were noticed.

In male controls, both at day 0 and day 49, minimal glomerular abnormalities were present, which were absent in female controls. In male LOU/M Wsl rats the nephropathy continued to develop steadily and sharply, whereas in female rats the increase of nephropathic lesions was less pronounced and the severity of nephropathy at the end of the experiment stood behind that of males. A compilation of data is presented in table 3. The mean nephropathy score is shown in Text-figure 2.

TABLE 3

Nephropathy score, assessed by glomerular changes, in male and female Lou/M Wsl rats by time and cumulative dose doxorubicin

Day	Cumulative dose mg/kg	Scores				
		0	+	++	+++	n[1)]
male						
0	0	1	3			4
10	5	4	1			5
14	6		4	1		5
21	7		1	3		4
28	8			4	1	5
35	9			3	2	5
42	10			1	4	5
49	11				4	4
49	0					2
female						
0	0	4				4
10	5	4				4
14	6	4	1			5
21	7	3	2			5
28	8		4	1		5
35	9	2	2	1		5
42	10		5			5
49	11		1	4		5
49	0	1	1			2

1) n = number of animals

Ultrastructural studies were done on kidneys of doxorubicin treated rats and controls, both males and females on day 35. In control animals no abnormalities were observed. Major lesions were found in the glomerular epithelium of doxorubicin treated animals. A striking change was the remarkable disappearance of all foot processes of podocytes in such a way that nearly the whole basal lamina was covered with a continuous layer of flattened visceral epithelial cells (Figs. 6, 7). In addition the visceral cells and, the parietal cells to a lesser extent, showed a large number of cytoplasmic vacuoles. The small and medium sized vacuoles appeared to be dilated rough endoplasmic reticulum (RER), but the large vacuoles were surrounded by a smooth membrane (Figs. 8, 9). An increased number of villi were present on the surface of the visceral epithelial cells and numerous microfibrils were present in their cytoplasm (Fig. 10). Finally the glomerular basement membrane was focally thickened and sometimes multilayered. Occasionally large areas were completely occupied by tortuous and thickened layers of basement membranes. In the cytoplasm of the visceral epithelial cells some cross-striated fibrils were observed, running parallelly and next to the glomerular basement membrane.

In female rats the lesions were less pronounced. No ultrastructural differences were observed in proximal tubuli between doxorubicin treated rats and control rats, both male and female rats.

Fig. 1. This section shows subtle strings between the parietal and visceral leave of Bowman's capsule (arrow). In addition few swollen nuclei of the epithelium of the parietal leave (arrowhead). This lesion was scored grade +.
1 µm 2-hydroxyethyl methacrylate embedded section. Giemsa x 320.

Fig. 2. This section shows a row of swollen epithelial nuclei of the parietal leave of Bowman's capsule (arrow). This lesion was scored grade +.
1 µm 2-hydroxyethyl methacrylate embedded section. Giemsa x 320.

Fig. 3. A grade ++ glomerular lesion on the right next to a normal glomerulus demonstrating the focal character of glomerulopathy in part of the material.
1 µm -2hydroxyethyl methacrylate embedded section. Giemsa x 320.

Fig. 4. The basal membrane of the parietal leave of Bowman's capsule is thickened (arrow). Pseudovacuoles have been formed (asterisk). This glomerulus is only partly affected, and presents an example of segmental glomerulopathy. This lesion was scored as grade ++.
1 µm 2-hydroxyethyl methacrylate embedded section. Giemsa x 320.

Fig. 5. Pronounced thickening of the basal membrane has taken place. The glomerular structure is severely damaged. Note the mitotic figure (arrow). Protein cast are present in tubuli. This lesion was scored grade +++.
1 µm 2-hydroxyethyl methacrylate embedded section. Giemsa x 320.

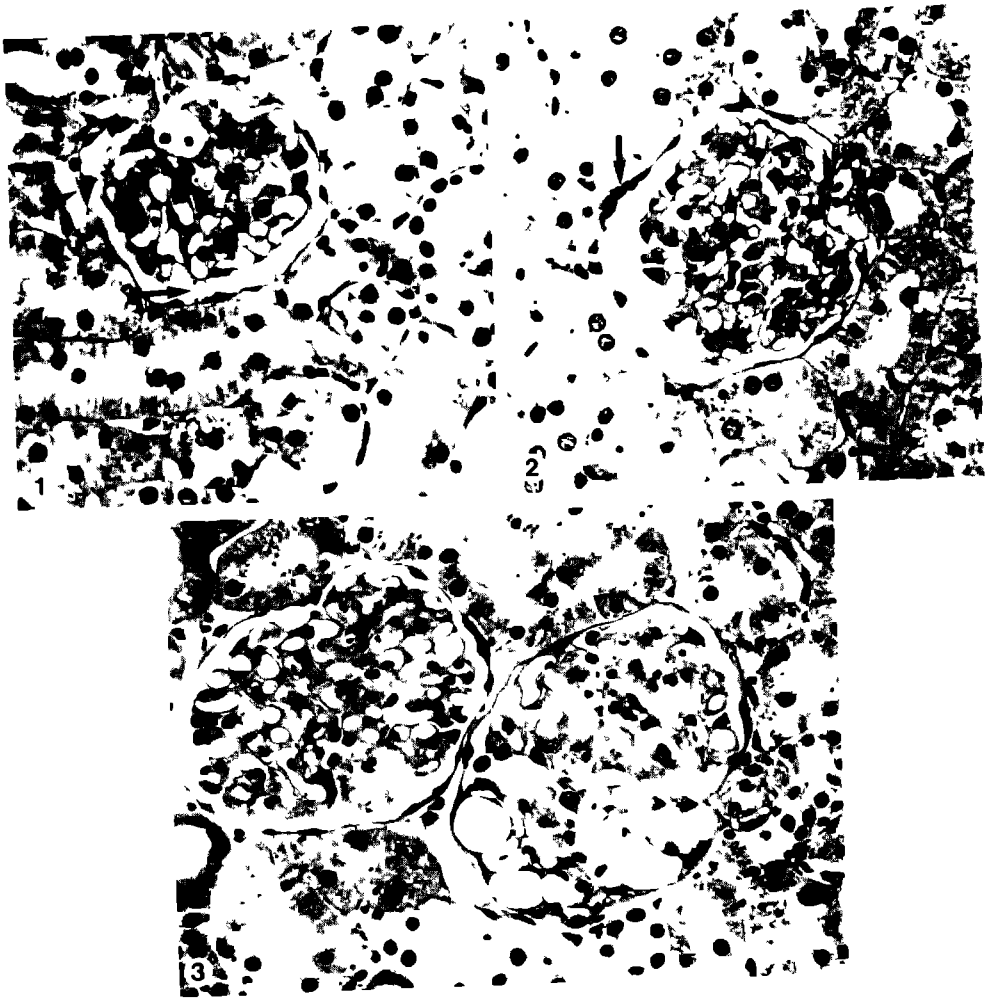
Fig. 6. Detail of control glomerulus showing some podocytes with their distinct foot processes (arrow), attached to the basal lamina. x 4950.

Fig. 7. Detail of glomerulus of doxorubicin treated rat. The basal lamina is covered by a nearly continuous layer of epithelial cell processes (arrow). Some vacuoles are present in the epithelial cells (asterisk). x 7650.

Fig. 8. Detail of glomerular podocyte of doxorubicin treated rat with dilated cisterns of rough endoplasmic reticulum. x 13.800.

Fig. 9. Large vacuoles, surrounded by a smooth membrane, are present in a glomerular podocyte of a doxorubicin treated rat. x 9500.

Fig. 10. Numerous villi are covering the surface of the visceral epithelial cells of a doxorubicin treated rat. x 6500.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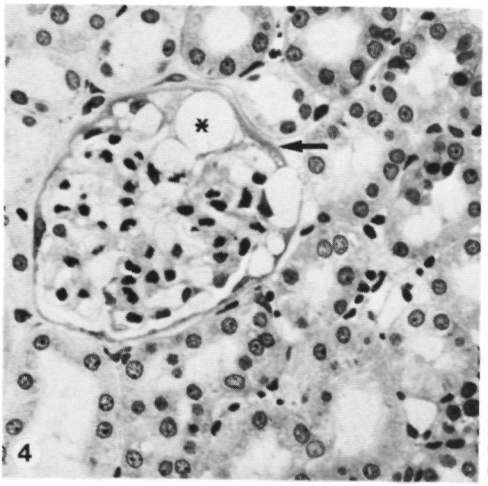
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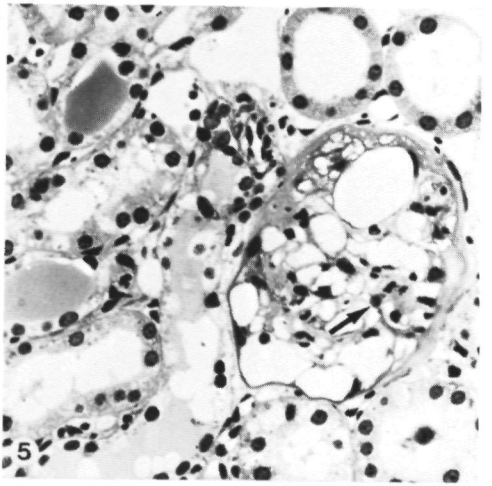
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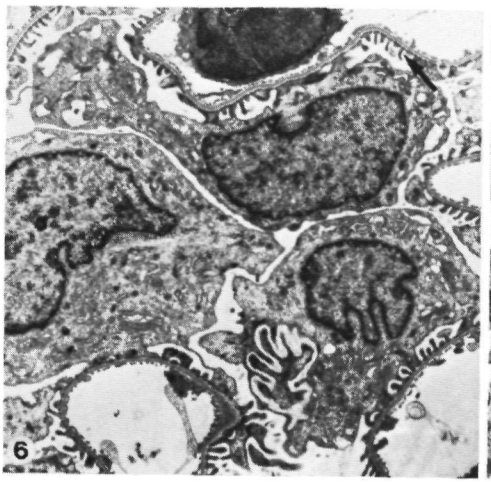
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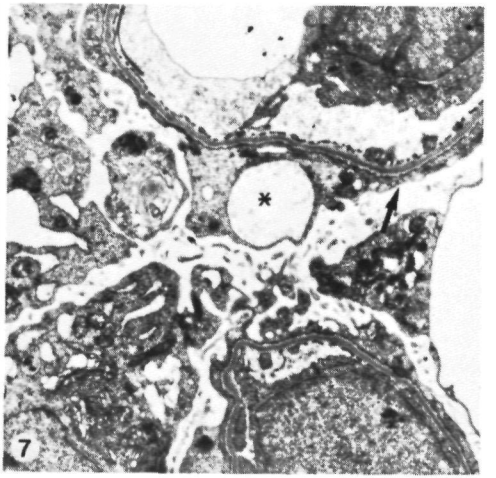
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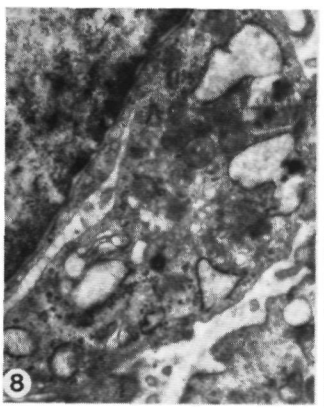
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In male and female LOU/M Wsl rats spontaneous albuminuria at the 0.3 - 1.0 g/liter level is present. After administration of doxorubicin albuminuria increases. Albuminuria has been shown to be dose dependent (1, 2). In this study male rats show at day 14 urinary albumin concentrations above 10 g/liter, while female rats reach this level more gradually yet at day 49. The urinary albumin scoring system is logarithmical by nature. The urinary albumin losses in male rats should therefore be considered considerable. As a consequence, the serum albumin levels in male rats decrease sharply. In contrast, the female rats are able to maintain their serum albumin level until day 42. Modification of the doxorubicin administration schedule did not prevent severe albuminuria. This indicates that variation of time intervals between subsequent doxorubicin administrations do not prevent doxorubicin induced toxicity. Albuminuria grade 4 is consistently present after day 30, the cumulative doxorubicin dose varying between 4 mg/kg and 8 mg/kg. These data suggest that time is an important factor in the expression of nephropathy.

In contrast to the major disturbances in renal handling of albumin, particularly in male rats, serum creatinine levels are relatively unaffected. Although in both sexes a gradual increase of serum creatinine levels is observed, the physiological meaning is not known. Based on the serum creatinine levels, the decrease of glomerular filtration rate is estimated to be 30% maximally. Although body weight did not change during the experiment (data not shown), in fact there may have been a significant loss of muscular mass, replaced by edema. In that case, serum creatinine levels have been flattered. Giroux et al. (10) found after a cumulative dose of 7 mg/kg free doxorubicin an increase in serum creatinine level of 0.92 ± 0.40 mg/dl to 1.40 ± 0.32 mg/ml, i.e. a decrease of creatinine clearance of 35% in their heminephrectomized rats. In our study, prolonged measurement of serum creatinine levels was impossible, due to turbidity of serum. The turbidity was ascribed to hyperlipidemia. Bizzi et al. (11) found 14-21 days after a single dose of doxorubicin (7.5 mg/kg i.v.) hyperlipidemia in male CD-COBS rats. Because hyperlipidemia completes the criteria required in nephrotic syndrome, the doxorubicin induced nephrotoxicity can be described as nephrotic syndrome.

The nephropathy induced by doxorubicin in the LOU/M Wsl rat as seen by

light microscopy resembles the nephropathy described by Sternberg (4) for daunorubicin, Bertani et al. (5), and Weening et al. (12) for doxorubicin in their rat models. The lesions in our model are also in agreement with those described by Fajardo et al. (3) in the rabbit.

The doxorubicin induced ultrastructural effects, as seen in this study, were identical with those in the rat reported by Bertani et al. (5). They supposed that the morphological changes and the proteinuria are the consequence of the loss of negative charge of the sialoproteins in the glomerular barrier by doxorubicin. Changing the electrolytic barrier of the glomerulus resulted in an enhanced filtration of albumin. In vivo perfusion experiments in the rat with polykations supported their hypothesis as the same morphological changes occurred, while perfusion with neutral macromolecules or polyanionic proteins induced no alterations (13).

The small and medium sized vacuoles clearly originated from dilated RER. It is well known that dilation of RER is accompanied by degranulation. Sternberg (4) described dilation of RER cisterns after daunomycin administration, while Fajardo et al. (3) too noticed a widespread dilation of RER after doxorubicin administration in the rabbit. Seiler et al. (13) hypothetized that retraction of foot processes also could result in a formation of urinary pockets between epithelial cell bodies and deformed foot processes.

In both sexes a steady increase in cardiomyopathy has been observed. No clear differences between male and female rats have been observed.

The heavy doxorubicin induced albuminuria in male LOU/M Wsl rats, which exceeds an urinary albumin concentration of 10 g/l and more on day 14 and thereafter, is at light microscopic examination associated with glomerular and tubular changes. The extent and severity of nephropathy increases steadily in time. Urinary albumin concentrations in female LOU/M Wsl rats are less than in males during the experiment until day 49. In parallel with this result, the findings at light microscopic examination of the kidney are less severe and the rate of increase is less steep in comparison with males. From a collaborative study by Julicher et al. (submitted for publication), it appeared that our findings on nephropathy parallelled biochemical data concerning parameters of free radical stress. In their study more loss of enzymatic activity in the kidney of male LOU/M rats was found as compared to females, after 52 days of treatment with doxorubicin on five consecutive days and then weekly. In heart tissue no

clues for damage of lipids or enzymes was found both in male and female LOU/M rats, whereas our study clearly describes histological damage in rats of both sexes. Julicher et al. conclude that peroxidation of lipids is not decisive in the development of cardiomyopathy.

Anthracycline induced nephropathy has been described relatively early in literature (4). In clinical practice no clues are present for doxorubicin induced nephropathy. Therefore, it is feasible that during the seventies no attention has been paid to the susceptibility of rats and rabbits for anthracycline induced nephropathy, and its possible interference with cardiac performance.

The onset of cardiomyopathy and nephropathy is simultaneous in both sexes. The severity of cardiomyopathy in male and female LOU/M Wsl rats is about the same in contrast to clear difference in nephrotoxicity. Moreover, the onset of independent cardiomyopathy and nephropathy is simultaneous in both sexes. In addition, the severity of cardiomyopathy in male and female LOU/M Wsl rats is about the same in contrast to clear difference in nephropathy. Therefore, it is concluded that cardiomyopathy observed in LOU/M Wsl rats is a phenomenon independent of nephropathy.

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Reduced cardiotoxicity and nephrotoxicity with preservation of antitumor activity of doxorubicin entrapped in stable liposomes in the LOU/M Wsl rat

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ABSTRACT

LOU M/Wsl rats inoculated s.c. with 10^4 IgM immunocytoma cells have a palpable tumor at day 17. Doxorubicin (DXR) has been entrapped in negatively charged liposomes (lip^- DXR) composed of egg-phosphatidylcholine (PC), cholesterol (chol) and phosphatidylserine (PS) and in positively charged liposomes (lip^+ DXR) composed of PC, chol and stearylamine (SA). DXR, lip^- DXR and lip^+ DXR were administered i.v. (0, 0.25, 0.5, 1.0 and 2.0 mg/kg) at day 17 for 5 consecutive days and then weekly. Control animals showed progressive tumor growth leading to death 27 days after inoculation. Antitumor activity for all three preparations was dose dependent. DXR and lip^- DXR showed the same antitumor activity, lip^+ DXR had less antitumor activity. The overall survival of tumor bearing animals treated with 2.0 mg/kg lip^- DXR was significantly prolonged ($p < 0.05$) in comparison to the animals treated with 2.0 mg/kg free DXR. Grade III cardiomyopathy was observed 47 days after treatment with free DXR; treatment with lip^- DXR resulted in grade I cardiomyopathy. In animals treated with 1.0 mg/kg and 2.0 mg/kg free DXR urinary albumin concentrations of 10 g/l were observed. Treatment with 1.0 mg/kg lip^- DXR and 1.0 mg/kg lip^+ DXR resulted in urinary albumin concentration of < 3.0 g/l and < 1.0 g/l, respectively. Free DXR 1.0 mg/kg resulted in a decline of serum albumin concentration from 27.8 ± 3.2 g/l to 9.6 ± 4.2 g/l. No such decline was observed after treatment with lip^- DXR and lip^+ DXR.

Treatment with 1.0 mg/kg dose of free DXR resulted in severe glomerular and tubular lesions which were not found after treatment with 1.0 mg/kg lip^- DXR and 1.0 mg/kg lip^+ DXR. Administration of lip^- DXR resulted in lower DXR levels in cardiac and renal tissue compared to administration of free DXR. After administration of lip^+ DXR very low tissue and tumor DXR levels were found.

In conclusion, treatment with lip^- DXR or lip^+ DXR resulted in a prolonged survival, less albuminuria, and higher serum albumin levels. Also less lesions in heart and kidney were found, correlating with lower DXR levels in these organs. Only lip^- DXR had the same antitumor effect as free DXR.

Doxorubicin is a very important antitumor agent with a wide spectrum of activity (4). Its clinical application is hampered by chronic cardiotoxicity, which incidence rises steeply above a cumulative dose of 550 mg/m^2 (18). Therefore current recommendations suggest that total cumulative doses should not increase above 550 mg/m^2. The chronic cardiotoxicity is morphologically characterized by myofibrillar loss, vacuolisation of the sarcoplasmic reticulum and ultrastructurally by swelling of mitochondria, disruption of mitochondrial crystae, and loss of tight junctions. In rabbits and rats, morphological and functional changes of the kidney have also been described (2, 7, 12, 31, 32).

To increase the therapeutic index of doxorubicin, several lines of research have been developed. The concomitant administration of anti-oxidants, e.g. α-tocopherol, has been studied in animal systems (6, 23, 30, 33) and has been clinically evaluated (20). The development and clinical testing of analogs is a major area of research (8). Further alteration of pharmacokinetic behavior designed to avoid high plasma DXR peak levels, has been subject of research. Decrease of the incidence of cardiotoxicity has been described after small weekly doses (34), and favorable results in clinical trials have been reported on continuous infusion (19, 21).

To improve the therapeutic index of DXR, entrapment of the drug into liposomes has recently drawn considerable attention. Liposomes are preferentially cleared from the plasma by tissues with a sinusoidal capillary system and by the mononuclear phagocyte system. After i.v. injection a high level of DXR, which was entrapped in liposomes, was found in the plasma, liver, spleen and lung compared to administration of free drug (15, 24, 27). This offers the opportunity to deliver relatively large amounts of cytostatic drugs to liver and lung, organs which are common sites of metastatic disease. Therefore, the usefulness of DXR entrapped in liposomes has been studied (13, 14, 15, 24, 27, 28, 29).

Thusfar, several mouse models have been reported in which the use of DXR entrapped in liposomes has shown similar or enhanced antitumor activity and reduced cardiomyopathy compared to free DXR (13, 14, 15, 24, 28, 29). Rahman found less antitumor activity for DXR entrapped in negatively charged liposomes (28). In all reports reduced cardiomyopathy was

accompanied by lower concentration of DXR in cardiac tissue. Moreover Forssen en Tökes (14) showed that there was no correlation between the absolute amount of drug delivered into the tumor and the level of antitumor activity.

Comparing the effectiveness of several kinds of liposomes, one finds conflicting results. Rahman et al. (28, 29) have reported on cardiac protection and identical antitumor activity obtained by administration of positively charged liposomes, whereas negatively charged liposomes did not preserve myofibrillar integrity and showed less antitumor activity against s.c. implants of Lewis lung carcinoma. In contrast, Olson et al. (24) and Forssen and Tökes (13, 14) reported on reduction of DXR induced cardiotoxicity and increased antileukemic activity with DXR entrapped into negatively charged liposomes. No general rule can yet be given for the type of liposome to be preferred.

The entrapment of DXR in liposomes might influence the kinetics of DXR in such a manner that not only toxicity, but also antitumor activity is diminished. Therefore it is extremely important to study at the same time antitumor activity and cardiomyopathy of liposome entrapped DXR in tumor bearing animals. We report here on the application of DXR entrapped in liposomes, with high stability and prolonged shelf life, which were well defined regarding charge, size, and polydispersity index, in the IgM immunocytoma bearing Lou/M Wsl rat (11, 32). In this study liposome entrapped DXR is compared to free drug in a multiple dosage regimen with respect to antitumor activity, survival, cardiotoxicity, nephrotoxicity, and tissue distribution of DXR.

Animals. Breeding pairs of LOU/M Wsl rats and the transplantable IgM immunocytoma of LOU/C Wsl origin were kindly provided by Dr.H.Bazin (Catholic University of Louvain, Belgium) (1). Animals were bred at the National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands. Male rats, weighing 170-220 g and 12 weeks of age were used. Animals were maintained according to accredited procedures in our facility and enjoyed uniformly good health at the initiation of the studies.

Tumor model. LOU M/Wsl rats were inoculated subcutaneously on the left flank with 1 x 10^4 IgM immunocytoma cells in 0.5 ml plain RPMI-1640 medium (Gibco, Europe B.V. Hoofddorp, The Netherlands). Details on the tumor model are described elsewhere (32). The growth of the tumor was measured twice a week with vernier callipers and expressed as mean value of three perpendicular measurements. Most animals inoculated with 1 x 10^4 cells developed a palpable tumor after 17-21 days, which grew to a diameter of 25-35 mm within 6-7 days. At this time the tumor has metastasized to the regional lymph nodes and micrometastases in the liver can be detected (11).

Drug preparation. Doxorubicin (Adriablastine^R) was purchased from Laboratoire Roger Bellon S.A., Neuilly sur Seine, France. For experiments with the free drug, the lyophylized powder of 10 mg/vials was reconstituted and diluted with sterile saline 0.9% w/v.

Preparation of liposomes. The chemical composition of the liposomal bilayers was adapted from Rahman et al. (28, 29). Positively charged liposomes consisted of egg yolk alpha-L-phosphatidylcholine (PC), type V-E (Sigma Chemicals, St.Louis, Miss., USA), cholesterol (chol) (Sigma Chemicals) and stearylamine (SA) (ICN Pharmaceuticals, Cleveland, Ohio, USA) in the molecular ratio 10 : 4 : 3. For negatively charged liposomes the molecular ratio of PC : chol: bovine brain phosphatidylserine (PS) (Sigma Chemicals, St.Louis, Miss., USA) of 10 : 4 : 1 was used. DXR from commercially available vials, which contain lactose, was mixed with PC, cholesterol and SA or PS in 5 ml of a chloroform/methanol mixture 1 : 1

(reagent grade). The dispersion of positively charged liposomes were sonicated (Bransonic B12, probe type sonicator, Stanford, Conn., USA) in consecutive 2 minute bursts separated by an one minute rest interval. The multilamellar structures were removed by ultracentrifugation at 10^5 g for one hour (4^oC). Negatively charged liposomes were extruded sequentially through Nucleopore membrane filters with pore sizes of 600 and 200 nm. Separation of free from liposome bound DXR was achieved by dialysis. The dispersions with the positively or negatively charged liposomes contained 0.2 mg/ml and 0.5 mg/ml DXR, respectively. Freshly prepared liposomal preparations were administered. Details of the preparation procedure are reported elsewhere (9, 10). Particle size was determined by dynamic light scattering (Nanosizer, Coulter Electronics, Ltd., Lutton, U.K.). One day after ultracentrifugation positively charged liposomes with DXR had a mean diameter of 0.10 μm. During dialysis this mean diameter and, in particular, the polydispersity of the liposomes tended to increase. After dialysis a mean diameter of 0.12 μm was found. The reproducibility of the mean liposome diameter of the extruded negatively charged liposomes was 0.27 ± 0.01 μm (n = 11). The polydispersity index was low. These data did not change during dialysis or storage.

The zeta-potential of the liposomes was determined by microelectrophoresis (Rank Brothers Mark II, Bottisham, U.K.). For lip^+ DXR a zeta-potential of 22 mV was calculated. The zeta-potential for lip^- DXR was found to be -9 mV.

Experimental design. Fourteen groups consisting of 6 animals each were formed at random. Seventeen days after tumor cell inoculation treatment was started. The tumor diameter was 18.1 ± 3.1 mm. For data relating to administration of drug the first day of i.v. injection was assigned day 0. I.v. injections were performed on five consecutive days and then weekly. The following dosages were used: saline 0.9%, 0.25, 0.5, 1.0, and 2.0 mg/kg free DXR; empty lip^-, 0.25, 0.5, 1.0 and 2.0 mg/kg DXR in lip^-; empty lip^+, 0.25, 0.5 and 1.0 mg/kg DXR in lip^+. The 2.0 mg/kg lip^+ DXR dose was not applied. Due to the relatively very low DXR content in positively charged liposomes, we were not able to produce a sufficient amount of lip^+ DXR to perform this part of the experiment.

The animals were weighed once weekly. Tumor measurements were done twice weekly. Albuminuria was assessed weekly. Blood was obtained weekly

by orbital puncture. Sera were stored at -20° C for determination of albumin and creatinine levels.

To compare the severity of the cardiomyopathy and nephropathy induced by free or liposome entrapped DXR, all animals of the same dosage groups were killed as soon as one animal of the free DXR group (1.0 or 2.0 mg/kg) developed clinically conspicious ascites or became moribund. In this way fresh tissues of animals treated with an equal accumulative dose were obtained for histological examination.

Survival study. For studies on survival 30 animals were randomly distributed in three groups. All animals were inoculated with 1 x 10^4 IgM immunocytoma cells on the left flank. On day 21 the tumor had reached a diameter of 20 mm; on this day treatment was started. In the first group, which was treated with saline 0.9% w/v, one animal died intercurrently before treatment was initiated. The second group received 2.0 mg/kg DXR, the third group 2.0 mg/kg lip^- DXR. Intravenous injections of saline or drug were given on five consecutive days and then weekly. Mortality was scored. No additional data were collected in this part of the study.

Histopathology. Tissue of the heart and kidneys was fixed in 4% buffered formaldehyde and embedded in 2-hydroxyethyl methacrylate. One µm sections were stained with Giemsa and examined with a light microscope. The lesions in heart tissue were scored according to Billingham (3).

Biochemistry. Albuminuria was assessed with Albustix (Ames, Division of Miles Nederland, Weesp, The Netherlands). The serum total protein, serum albumin, and serum creatinine levels were determined in an automatic device (Cobas Bio, Roche, Basel, Switzerland).

Tissue distribution studies

For determination of DXR tissue distribution three groups of 12 tumor bearing rats each were formed. Four and 24 h after administration of 1 mg/kg DXR, 1 mg/kg lip^- DXR, and 1 mg/kg lip^+ DXR respectively, 6 animals were killed. Plasma, heart, kidney, lung, liver, spleen, quadriceps musculature and tumor were collected and stored at -20° C until analysis. Plasma was analysed according to the method described previously (5).

For determination of DXR in organs, tissues were freeze-dried as whole

organs during 24 hours and then finely powdered and homogenized in a mortar. Powdered samples of 100 mg, accurately weighed, were transferred into a 15 ml polypropylene tube. Then, 100 µl of a daunorubicin (Specia, Paris, France) internal standard solution (4 mg per liter distilled water) and 500 µl silver nitrate solution (330 g per liter distilled water) were added. The mixture was vortexed for 10 seconds and after addition of 500 ul sodium chloride solution (2 M in distilled water) vortexed again during 10 seconds. After adjusting the pH = 9.0 by means of 3.0 ml of the borax buffer solution pH = 9.0 (53.5 g of borax per liter distilled water containing approximately 70 ml 1M HCl) 3.0 ml of a chloroform-1-heptanol mixture (1 : 1) was added. The mixture was shaken for 10 minutes and then centrifuged for 10 minutes at 2000 g. The aqueous upper layer was removed and the organic layer was transferred into a 5 ml polypropylene tube containing 300 µl 0.1 M phosphoric acid. After 1 min. vortexing and 10 min. centrifugation at 2000 g, 100 µl of the phosphoric acid phase was injected into the chromatographic system. The system consisted of a model 6000, a solvent delivery system and a WISP 710 A automatic injection system (both from Waters Assoc., Milford, MA., USA). The detector was a Perkin Elmer (Norwalk USA) Model 204 fluorescence detector equipped with a 25-µl flow cell (Hellma GmbH, type 176.70, Müllheim Baden GFR). Fluorimetric detection was performed at excitation wavelength 470 nm and emission wavelength 562 nm. A LiChrosorb RP-8 (Hibar 125-4, Merck, Darmstadt, FGR) reversed phase column (125 mm x 4 mm I.D., particle size 5 µm) was used in combination with a pellicular reversed phase filled guard column (75 mm x 2.1 mm I.D., Chrompack, Middelburg, The Netherlands). The mobile phase consisted of acetonitrile-distilled water - 0.1 M phosphoric acid (42 : 55 : 3) containing 10 µg desipramine-HCl per ml and was filtered through a 0.22 µm filter under reduced pressure before use. The flow rate was 2 ml/min. All chromatographic analyses were performed at room temperature. The reagents were of analytical grade and used without further purification.

Calibration curves were constructed by adding known amounts of standards to pooled, freeze-dried, finely powdered, homogenized, non treated rat tissues. Tissue DXR content was calculated from the ratio between the areas of the fluorescence of the internal standard and the tissue extracted DXR. During each run of the automatic injector, four standards were injected too. Simultaneously with each series of rat tissues the standards

were subjected to the extraction procedure.
With this method linear results were obtained to at least 20 mg per kg freeze-dried tissue. The detection limit was 0.1 mg/kg. Calibration curves for the analysis of spiked, pooled, freeze-dried non-treated rat tissues were linear over the range 0.2 mg/kg to 20 mg/kg and pass through the origin (r^2 = + 0.999). Tissue concentrations exceeding the studied upper limit were re-analysed by weighing an appropriate amount of freeze-dried tissue powder.

Statistics. Differences in group means were analysed by Student's t-test (two-sided). In case of insufficient homogeneity of variances, the Welch correction with respect to the degrees of freedom was applied.

Antitumor activity (Chart 1). Administration of saline 0.9% w/v, empty negatively or positively charged liposomes did not inhibit tumor growth. Doses of 0.25 mg/kg free DXR, lip^- DXR, and lip^+ DXR retarded tumor growth. At days 7, 11 and 14 the differences between 0.25 mg/kg free DXR versus lip^- DXR and lip^+ DXR were statistically significant ($p < 0.05$). 0.5 mg/kg Free DXR and lip^- DXR resulted temporarily in regression of tumor, however the antitumor activity of free DXR was superior at day 18 ($p < 0.05$), 21 and 25 ($p < 0.01$) and 28 ($p < 0.05$). Lip^+ DXR 0.5 mg/kg had only a retarding effect on tumor growth. Tumor regression was seen in all animals treated with 1.0 mg/kg, but was least pronounced in animals treated with lip^+ DXR. No significant differences in the mean tumor diameter were found between free DXR and lip^- DXR . Regrowth of tumor was seen in all groups. The 2.0 mg/kg free DXR and 2.0 mg/kg lip^- DXR groups showed a nearly complete regression of tumor, but because of emaciation of the free DXR group, both groups were killed on day 14.

Body weight. Treatment with 1.0 mg/kg dose of free DXR resulted in a decrease of body weight of 8.1% within 10 days after the start of drug administration. Body weight diminished 4.1% in 1.0 mg/kg lip^- DXR group and 3.2% in 1.0 mg/kg lip^+ DXR treated groups. The difference between DXR and lip^- DXR was statistically not significant. In the lip^- DXR group, however, twenty-eight days after the start of therapy, a rise of bodyweight was observed to a level 10% above the weight on the first day of treatment. This rise could not be correlated with the development of ascites.

A very steep decline (-40%) of body weight was observed in the 2.0 mg/kg free DXR group. In contrast, the 2.0 mg/kg lip^- DXR group lost 10% of their bodyweight during the first 14 days of treatment ($p < 0.001$). The same pattern was seen in the survival experiment.

Ascites. Ascites was observed when serum albumin levels had dropped below 10.0 g/l. After a cumulative dose of 12.0 mg/kg DXR it was observed on day 14 in the 2.0 mg/kg free DXR group. The first sign of ascites in the 1.0 mg/kg group was observed on day 47, after a total cumulative dose of 10 mg/kg free DXR. In contrast, none of the groups treated with lip^- DXR and lip^+ DXR developed ascites.

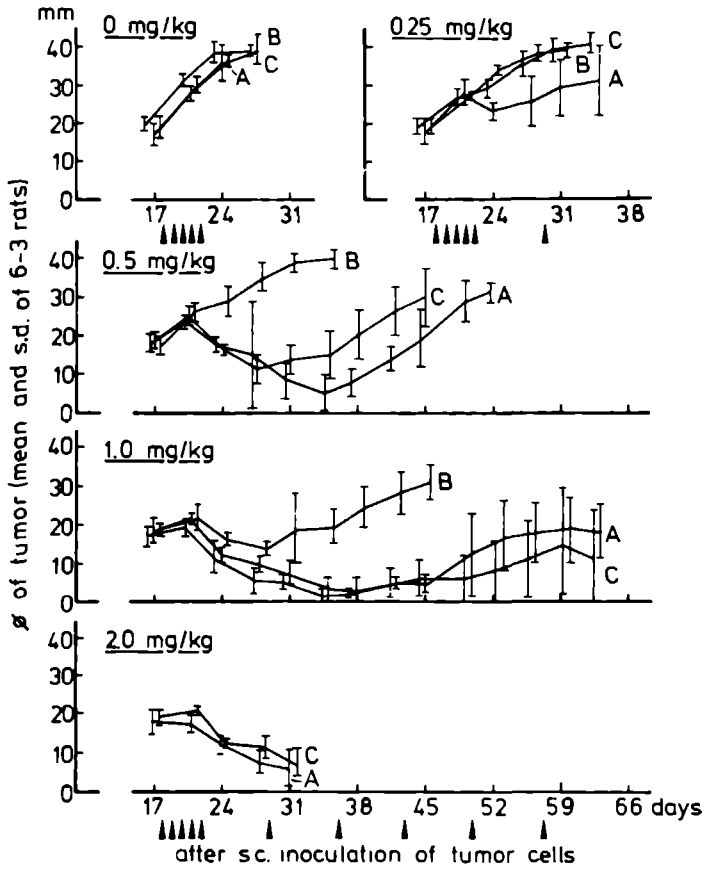

Chart 1. Effect of liposome-entrapped DXR on growth of solid IgM immunocytoma in LOU/M Wsl rats. ▲:i.v. injection; A, DXR in saline; B, DXR in lip^+ DXR; C, DXR in lip^- DXR; ø diameter, mean value of 3 perpendicular measurements. Bars, S.D.

Table 1.

Effect of free doxorubicin (DXR) and doxorubicin entrapped in negatively (lip^- DXR) or positively (lip^+ DXR) charged liposomes on cardiomyopathy of tumor bearing animals.

doxorubicin dosage mg/kg		cardiomyopathy score	number of animals	day of assessment
	saline 0.9%	0	3	
	empty lip^- DXR	0	3	10
	empty lip^+ DXR	0	2	
0.25	DXR	0	5	
	lip^- DXR	0	5	17
	lip^+ DXR	0	2	
1.0	DXR	III	4	
	lip^- DXR	I	4	47
	lip^+ DXR	I	2	
2.0	DXR	I	5	
	lip^- DXR	I	4	14
	lip^+ DXR	ND	-	

[a] Grading system according to Billingham (3)

[b] Day of assessment day 33

[c] ND = not done

Histology

Heart. As shown in Table 1, the saline, empty negatively charged or positively charged liposome treated animals showed no histologic abnormalities of the heart. Neither were myocardial lesions seen in the 0.25 mg/kg groups, which survived 17 days after the start of drug administration. Animals of all 0.5 mg/kg dose groups died because of tumor

growth without obtaining fresh tissue samples for histologic examination. 47 Days after the initiation of therapy the 1.0 mg/kg free DXR treated group had developed vacuolisation and lysis of myofibrils diffusely throughout the myocardium, to grade III cardiomyopathy according to Billingham (3). Animals treated with 1.0 mg/kg lip^- DXR, which were killed at the same day showed only sporadic myocardial lesions, to grade I myocardiopathy. Assessment of cardiomyopathy in the animals treated with 1.0 mg/kg dose of lip^+ DXR took place on day 33, because tumor growth necessitated killing of the animals. In this group grade I cardiomyopathy was found. Both 2.0 mg/kg free DXR and lip^- DXR treatment groups showed on the 14th day after the start of the therapy of the drug sporadic myocardial lesions, scored as grade I cardiomyopathy.

Kidney. The control and 0.25 mg/kg dose groups showed no glomerular and tubular abnormalities. Treatment with 1.0 mg/kg free DXR resulted in extensive glomerular lesions as described previously (32). Synechial connections between the parietal and visceral leaves of Bowman's capsule, suggesting the existence of large vacuoles, were striking. Some glomeruli were infiltrated with many mononuclear cells, other ones were rather depleted of cells. The brush border of the proximal tubules was lost. The proximal, distal and collecting tubules showed anisocytosis and polymorphism of their nuclei. Mitotic figures were seen. Numerous protein casts were present in the collecting tubules. In contrast, the glomeruli of 1.0 mg/kg lip^- DXR treated animals showed only sporadic septate connections between the parietal and visceral leaves of Bowman's capsule. No lesions were noted in the 1.0 mg/kg lip^+ treated animals.

The short living 2.0 mg/kg free DXR treated group showed slight to moderate glomerular lesions. The 2.0 mg/kg lip^- DXR treated animals showed only sporadic glomerular lesions.

Albuminuria. The urinary albumin concentration at the start of therapy was in the 0.1 - 0.3 g/l range. Treatment with free DXR (1.0 and 2.0 mg/kg) resulted in a rapid increase of urinary albumin to a concentration above 10 g/l. Treatment with lip^- DXR resulted in urinary albumin concentration to levels < 3.0 g/l, whereas treatment with lip^+ DXR 1.0 mg/kg limited urinary albumin concentration to < 1.0 g/l. A compilation of data is given in Table 2.

Table 2. Effect of DXR, lip$^-$ DXR and lip$^+$ DXR on urinary albumin concentration of tumor bearing animals.

doxorubicin dosage mg/kg	days after starting treatment	urinary albumin concentration free DXR	lip DXR	lip DXR
0.5	0	< 0.3	< 0.3	< 0.3
	14	< 3.0	< 3.0	< 3.0
	21	< 3.0	< 1.0	
	28	< 3.0	< 3.0	
1.0	0	< 0.3	< 0.3	< 0.3
	14	< 3.0	< 1.0	< 1.0
	21	>10.0	< 1.0	< 1.0
	28	>10.0	< 3.0	< 1.0
	47	>10.0	< 3.0	–
2.0	10	>10.0	< 3.0	ND

[a] Urinary albumin concentrations were assessed by Albustic and expressed in g/l. ND = not done. Number of observations; 6-3 animals.

Serum albumin levels (Table 3). In the 1.0 mg/kg free DXR group a gradual decrease of serum albumin level was observed, whereas in the 2.0 mg/kg free DXR group a very steep decline was seen. Treatment with lip$^-$ DXR 1.0 mg/kg and 2.0 mg/kg preserved serum albumin levels. In the forementioned dosage groups treatment had resulted in relevant tumor regression.

Animals, which received no DXR, showed a decline in serum albumin level. The same was observed in all 0.5 mg/kg dosage groups, and in the lip$^+$ DXR 1.0 mg/kg group. All these groups are characterized by the presence of a huge tumor mass, which might interfere with protein metabolism.

Serum creatinine levels. Treatment with free DXR, lip$^-$ DXR or lip$^+$ DXR did not result in significant change in serum creatinine levels compared to the serum levels before treatment. Initial and end point values are shown in Table 4.

Table 3

Effect of doxorubicin and doxorubicin entrapped in negatively (lip^- DXR) or positively (lip^+ DXR) charged liposomes on serum albumin levels of tumor-bearing animals.

doxorubicin dosage mg/kg	days after starting treatment	Serum albumin level: free DXR	p value[a]	lip^- DXR	p value	lip^+ DXR	p value
0	0	29.9 ± 3.2[b]		28.9 ± 2.3		28.0 ± 1.8	
	7	21.0 ± 2.1	< 0.001	20.9 ± 1.5 (5)	< 0.001	21.6 ± 1.2	< 0.001
	10	19.5 (2)		20.8 ± 2.2	< 0.001	20.7 ± 0.6 (3)	< 0.001
0.5	0	29.4 ± 1.9		29.4 ± 1.1 (5)		28.3 ± 3.1	
	17	31.7 ± 1.1	< 0.05	30.2 ± 2.0 (5)	NS[c]	19.4 ± 3.9 (4)	< 0.05
	25	32.3 ± 1.5	< 0.05	26.5 ± 1.3 (5)	< 0.01[d]		
	33	22.4 ± 2.3	< 0.001	20.5 ± 0.7 (2)			
	40	17.5 ± 0.7 (2)	< 0.001				
1.0	0	27.8 ± 3.2		26.8 ± 2.9		28.9 ± 1.5	
	17	24.1 ± 3.1	NS	30.1 ± 0.7	< 0.05[d]	28.9 ± 2.6	NS
	25	20.1 ± 6.9	< 0.05	31.3 ± 1.5	< 0.05[d]	24.4 ± 3.6	< 0.05[d]
	33			28.5 ± 4.0		17.5 ± 4.2 (3)	
	40	12.4 ± 5.3	< 0.001	26.8 ± 4.5 (5)			
	47	9.6 ± 4.2 (4)	< 0.001	26.5 ± 5.6 (4)			
2.0	0	28.8 ± 1.4		27.7 ± 1.6		ND	
	7	24.5 ± 2.1	< 0.01	27.7 ± 1.5 (5)	NS		
	10	8.7 ± 4.0	< 0.001	28.9 ± 1.9 (5)	NS		

[a] According to Student's t-test, 2-tailed, compared to Day 0
[b] Mean ± S.D. serum albumin in g/liter.
[c] NS, not significant compared to Day 0; ND, not done.
[d] After Welch correction.

Table 4

Effect of doxorubicin and doxorubicin entrapped in negatively (lip^- DXR) or positively (lip^+ DXR) charged liposomes on serum creatinine level of tumor-bearing animals.

doxorubicin dosage mg/kg	days after starting treatment	Serum creatinine level: free DXR	p value[a]	lip^- DXR	p value	lip^+ DXR	p value
0.5	0	60.0 ± 9.8[b]		56.4 ± 10.4 (5)		48.5 ± 5.3	
	17	52.2 ± 2.1	NS[c]	47.2 ± 5.5 (5)	NS	63.2 ± 11.9 (4)	< 0.05
	33	72.0 ± 15.2	NS	50.5 ± 10.6 (2)	NS	ND	
	40	61.0 ± 1.4 (2)	NS	ND		ND	
1.0	0	54.3 ± 4.3		49.0 ± 4.4		53.5 ± 10.2	
	33	41.6 ± 5.8 (5)		50.3 ± 5.1		57.3 ± 5.1 (3)	NS
	47	50.0 ± 10.2 (4)	NS	52.8 ± 5.4 (4)	NS	ND	
2.0	0	56.5 ± 6.7		55.5 ± 10.5		ND	
	17	126.6 ± 73.1 (5)	NS[d]	49.4 ± 1.5 (5)	NS	ND	

[a] According to Student's t-test, 2-tailed, compared to Day 0.
[b] Mean ± S.D. of serum creatinine levels are expressed in µmol/liter.
[c] NS, not significant (compared to Day 0); ND, not done.
[d] After Welch correction.

Survival experiment. Since in the above described study the animals of corresponding groups were killed at the same time to obtain fresh tissues for histologic examination, a separate experiment was done to assess the survival time in the 2.0 mg/kg group. The saline treated animals showed a median survival time of 8 days (range 5-11) after start of therapy. The 2.0 mg/kg DXR treated animals survived for 23 days (range 11-33 days) after initiation of therapy. The 2.0 mg/kg lip^- DXR treated group survived considerably longer: median 40 days (range 17-65 days) (Chart 2). In both the 2.0 mg/kg free DXR and lip^- DXR treated group complete regression of tumor without recurrence occurred (Chart 3). Ascites was observed on day 22 (4 out of 5 animals) in the free DXR group. The animals treated with lip^- DXR had developed ascites 12 days later.

Serum and tissue doxorubicin content (Table 5)

The administration of 1.0 mg/kg lip^- DXR resulted 4 hours after i.v. injection in a plasma DXR level, which was significantly lower than the plasma DXR level after administration of free DXR. However, administration of lip^+ DXR, resulted in a very high plasma DXR level. Twentyfour hours after i.v. administration of free DXR the plasma DXR level was still slightly higher, compared to the plasma level after administration of lip^- DXR. No doxorubicinol could be detected in any plasma sample.

Both in cardiac tissue and in the kidney DXR levels at 4 hr after i.v. administration of lip^- DXR and lip^+ DXR were lower than after i.v. administration of free DXR. Twentyfour hours after administration the same pattern persisted.

DXR entrapped in negatively charged liposomes preferentially accumulated in liver and spleen, where at 4 and 24 h after i.v. administration very high tissue concentrations were found. Administration of DXR in positively charged liposomes resulted in tissue DXR levels in liver and spleen which were lower than after i.v. administration of free DXR at 4 h. Twentyfour hours after administration of lip^+ DXR no significant difference was observed.

In tumor tissue the highest DXR tissue levels were found after administration of free DXR. Administration of lip^- DXR resulted in lower tumor tissue levels, statistically significant ($p < 0.05$) at 4 h but not at 24 h. DXR levels in tumor tissue were higher 24 h after i.v. administration of lip^- DXR compared to the 4h levels. Very low DXR levels in tumor tissue

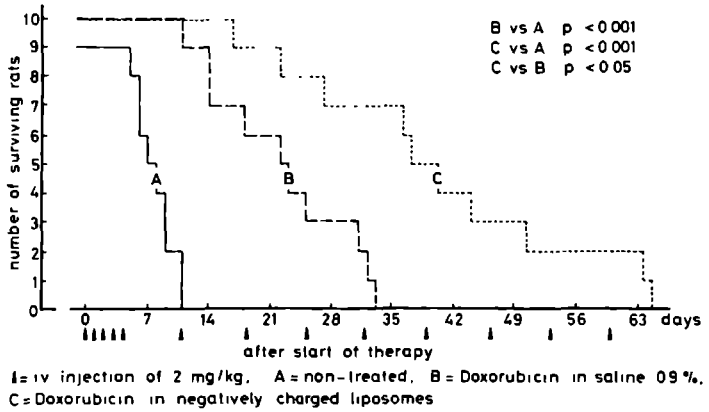

Chart 2. Survival of LOU/M rats bearing solid IgM immunocytoma. ▲, i.v. injection of 2 mg/kg; A, non-treated; B, DXR in saline; C, DXR in lip⁻.

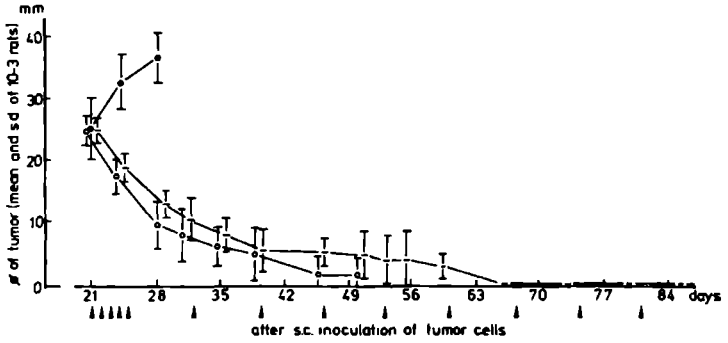

Chart 3. Effect of liposome-entrapped DXR on growth of solid IgM immunocytoma in LOU/M Wsl rats. ▲, i.v. injection of 2 mg/kg; A, nontreated; B, DXR in saline; C, DXR in lip⁻; O, diameter, mean value of 3 perpendicular measurements. Bars, S.D.

Table 5

Plasma and tissue concentration of DXR 4 hours and 24 hours after one i.v. injection of free, lip$^-$ DXR and lip$^+$ DXR (1 mg/kg) in IgM immunocytoma bearing rats.

	DXR levels							
4 hours	Free DXR	p value[a]	lip$^-$ DXR	p value	lip$^+$ DXR	p value	Free DXR	
Plasma	5.68 ± 0.56[b]	< 0.001	2.04 ± 0.50 (5)	< 0.001	35.0 ± 4.06	< 0.001	5.68 ± 0.56	
Heart	6.72 ± 0.67[c]	< 0.001	2.53 ± 0.43	< 0.001	0.74 ± 0.26	< 0.001	6.72 ± 0.67	
Kidney	12.97 ± 1.00	< 0.001	4.41 ± 0.67	< 0.001[d]	1.26 ± 0.51	< 0.001	13.0 ± 1.0	
Muscle	2.18 ± 0.28	< 0.001	0.96 ± 0.42	< 0.05[d]	0.29 ± 0.08	< 0.001	2.18 ± 0.28	
Lung	6.50 ± 0.84	< 0.001	3.16 ± 0.67	< 0.01[d]	1.28 ± 0.15	< 0.001	6.50 ± 0.84	
Liver	3.76 ± 0.56	< 0.001	18.3 ± 2.5	< 0.001	1.44 ± 0.36	< 0.001	3.76 ± 0.56	
Spleen	4.53 ± 0.88	< 0.001	54.6 ± 12.0	< 0.001	2.63 ± 0.93	< 0.01[d]	4.53 ± 0.88	
Tumor	2.12 ± 0.65	< 0.05	1.40 ± 0.34	< 0.001	0.32 ± 0.08	< 0.01[d]	2.12 ± 0.65	
24 hours								
Plasma	2.84 ± 0.57 (5)	< 0.05[d]	2.03 ± 0.39		ND[e]		2.84 ± 0.57 (5)	
Heart	3.28 ± 0.86 (4)	< 0.01[d]	0.66 ± 0.37 (5)	NS	0.44 ± 0.25	< 0.01	3.28 ± 0.86 (4)	
Kidney	5.58 ± 1.10	< 0.001	1.76 ± 0.28 (5)	< 0.001	0.58 ± 0.04	< 0.001	5.58 ± 1.10	
Muscle	2.29 ± 0.37	< 0.001	1.27 ± 0.25	< 0.001	0.24 ± 0.05	< 0.001	2.29 ± 0.37	
Lung	5.35 ± 0.78 (4)	< 0.001	2.82 ± 0.53	< 0.001	0.99 ± 0.17	< 0.001	5.35 ± 0.78	
Liver	1.13 ± 0.10	< 0.001[d]	3.98 ± 0.98	< 0.001[d]	1.06 ± 0.42	NS	1.13 ± 0.10	
Spleen	4.02 ± 0.57 (4)	< 0.05[d]	30.2 ± 10.0 (4)	< 0.05	5.06 ± 1.89 (5)	NS	4.02 ± 0.57	
Tumor	2.81 ± 1.08	NS	2.04 ± 0.33	< 0.001	0.48 ± 0.17	< 0.01	2.81 ± 1.08	

[a] Comparing adjacent figures.
[b] Mean ± S.D. of serum DXR concentration expressed in µg/liter.
[c] Mean ± S.D. of tissue DXR concentration expressed in mg/kg freeze-dried tissue.
[d] After Welch correction
[e] NS, not significant; ND, not done.

were seen after i.v. administration of lip^+ DXR.

With exception of the spleen, doxorubicinol was not detectable in any tissue. Spleen levels of doxorubicinol approximated the detection limit and could not be quantitated accurately.

Our study showed antitumor activity for DXR entrapped in positively and negatively charged liposomes. However, the antitumor activity of DXR in negatively charged liposomes equalled the activity of free DXR, whereas the antitumor activity of DXR in positively charged liposomes at the three dosage levels investigated lagged behind the activity of free DXR. No antitumor activity by empty liposomes was seen. Simultaneous administration of negatively charged liposomes and non-entrapped DXR resulted in the same antitumor activity and survival as free DXR, underscoring the necessity of the entrapment of the cytostatic drug (data not shown). In comparison to free DXR, administration of lip^- DXR resulted in diminished toxicity: both heart and kidney structure were largely preserved. The free DXR related depletion of serum albumin did not occur. The decrease in body weight related to free DXR treatment was not significantly diminished by lip^- DXR. Delivery of DXR entrapped in negatively charged liposomes resulted in a prolonged survival time, and a higher cumulative dose of DXR, compared to administration of free drug.

The administration of DXR entrapped in negatively charged liposomes resulted in plasma DXR levels and tissue DXR distribution, which differed from the distribution obtained after i.v. administration of free DXR. Plasma, heart and kidney contained less DXR at 4 as well as at 24 hours after i.v. administration of lip^- DXR compared to i.v. administration of free DXR. This result parallels the finding of less signs of toxicity in heart and kidney on microscopical examination. In addition to the different DXR distribution, the favorable effects on myocardium and kidney structures might be explained by less direct exposition of this tissue to the drug entrapped in liposomes. In tumor tissue, 4 h after administration of lip^- DXR the DXR concentration is lower than after administration of free DXR. At 24 hours, however, there is no difference anymore. This might explain the fact that no difference in antitumor activity is observed.

The administration of lip^+ DXR resulted in a quite different pattern of DXR concentrations. A relatively high DXR level was found at 4 h in plasma. The analytical method does not discern between liposome entrapped DXR, protein-bound DXR, and free DXR, so nothing can be said about the clearance of positively charged liposomes laden with DXR. The very low

tissue levels, even in liver and spleen, indicate a low availability of DXR after entrapment in the positively charged liposomes. The lesser antitumor activity is readibly explained by the very low DXR concentration in tumor. Our results with negatively charged liposomes are in agreement with those of Forssen and Tökes (13, 14), Gabizon (15) and Olson et al. (24). However, both at 4 h and 24 h the plasma DXR level after administration of lip$^-$ DXR was lower than after administration of free DXR in our experiment, whereas the forementioned investigators found the contrary.

Rahman et al. (28) reported on the absence of cardioprotective properties, higher concentration x time DXR value and less antitumor activity for DXR entrapped in negatively charged liposomes (PC : Chol : PS = 50.6 : 20.7 : 5.03). However, entrapment of DXR in positively charged liposomes (PC : Chol : SA = 50.6 : 20.7 : 14.8) resulted in lower cardiac peak DXR concentration and lower cardiac DXR concentration x time value resulting in preservation of myocardial structure and in the same antitumor activity compared to free DXR. It may be that the different behavior is related to the species studied, or is related to differences in vesicle structure. Rahman (28) does not define the physical properties of his dispersions.

To obtain reproducible results it is of utmost importance to use well-defined liposomes, which can be made in a reproducible manner. Therefore we carefully checked particle size, charge, and physical and chemical stability of DXR containing vesicles (9, 10).

The relative cholesterol content influences the stability of a liposome (16, 25, 26). Despite the relatively low cholesterol content of our negatively charged liposomes, the stability has been satisfactory i.e. the leakage factor was low (10-15% after 60 days) and the shelf-life permitted the efficient production of larger quantities.

There may be three explanations for the observed reduced cardiomyopathy after administration of liposomes:

1. DXR entrapped in negatively charged liposomes is preferentially accumulated in the mononuclear phagocyte system. These cells might release DXR slowly into the plasma, accounting for prolonged and low plasma DXR levels, thus avoiding peak levels, which might initiate organ damage.
2. Liposomes might protect tissues susceptible to toxic effects of DXR by retaining DXR within their membranes during the distribution phase.
3. As a consequence of the preservation of renal integrity, serum albumin

levels do not decrease. This might indirectly have a favourable effect on myocardial function and structure, because hypoalbuminemia may have consequences for the circulation (32).

The mechanism of antitumor activity is unclear. Forssen and Tokes measured DXR concentrations in solid tumors, as we did (14). They found equal DXR levels in Sarcoma 180 after free DXR and DXR in the entrapped form. In Lewis Lung Carcinoma, however, lower levels of DXR were found after administration of the entrapped form, while antitumor activity was enhanced. It was concluded that a correlation between antitumor activity and tumor DXR level lacks. These data are giving rise to speculations about the mechanism of antitumor activity (14). Of interest are the in vitro studies of Haskill (17) and Martin et al. (22), about the transfer of DXR by macrophages to tumor cells. If this transfer takes also place in vivo, entrapment of DXR in liposomes might enhance the tumoricidal activity of macrophages. Functional studies of macrophages after in vivo and in vitro administration of liposome entrapped DXR are underway in our laboratory.

In conclusion, this study describes the use of DXR entrapped in liposomes in an IgM immunocytoma bearing rat. DXR entrapped in positively charged liposomes had less antitumor activity in comparison to free DXR and lip$^-$ DXR. As the negatively charged liposomes used in this study have a prolonged shelf-life of at least 60 days and a reduced cardiotoxicity and nephrotoxicity with preserved antitumor activity, studies on the suitability of these liposomes in clinical trials might be indicated.

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Chapter 8

The effect of entrapment of doxorubicin in negatively charged liposomes on hematological parameters in the male Lou/M Wsl rat

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SUMMARY

Doxorubicin (DXR) and doxorubicin entrapped in negatively charged liposomes (lip$^-$ DXR) which had shown to have beneficial effects on cardiomyopathy and nephropathy with preservation of antitumor activity in previous studies, have been i.v. administered to 12 week old male LOU/M Wsl rats in 1 mg/kg, and 2 mg/kg dose on 5 consecutive days. Saline, and empty liposomes have been administered as controls. Blood for hematological parameters and determination of cellular doxorubicin concentrations has been obtained by exsanguination at days 6, 9, 11, 13, 20, 34 and 48 in the 1 mg/kg experiment and on days 3, 6, 8, 10, 13 and 15 in the 2 mg/kg experiment.

White blood cell counts were depressed from day 3 to day 13, without significant differences between the free or liposome entrapped doxorubicin treated groups. Thrombocyte counts were depressed from day 6 to day 13. The depression was dose dependent. No difference between free DXR and lip$^-$ DXR treated groups was observed.

In the 1 mg/kg experiment no change in hemoglobin level was observed, in contrast to the 2 mg/kg experiment. No differences between the free or liposome entrapped doxorubicin treated groups were observed.

Flowcytometry has shown that after cessation of treatment cellular doxorubicin concentration in bone marrow cells steadily increased in the 2 mg/kg experiment, the fluorescence counts being higher in the liposome entrapped doxorubicin group, than in the free doxorubicin group. Despite

increasing doxorubicin concentrations bone marrow recovery set in.

In conclusion, the entrapment of doxorubicin in negatively charged liposomes had no benificial effects on doxorubicin induced suppression of bone marrow activity.

INTRODUCTION

Myelosuppression is the dose limiting toxicity for doxorubicin in every day practice (Bonadonna, 1970). On a long term, doxorubicin induced cardiomyopathy occurs. The incidence of cardiomyopathy rises steeply above a cumulative dose of 550 mg/m^2 (Lefrak, 1973). Prior radiation therapy and hypertension have been identified as risk factors (Minow 1977, Von Hoff 1979).

The encapsulation of doxorubicin in liposomes has been found to protect the animal in experimental systems against cardiac damage (Forssen 1981, 1983, Olson 1982, Rahman 1980, 1982, 1985, Van Hoesel 1984a). Besides cardiomyopathy, doxorubicin induces nephropathy, eventually resulting in a nephrotic syndrome, in rabbit and rat (Bristow 1981, Bertani 1982). Treatment with doxorubicin entrapped in liposomes reduces nephropathy and preserves serum albumin levels (Van Hoesel 1984a).

The administration of doxorubicin encapsulated in liposomes resulted in lower tissue doxorubicin concentration 4 and 24 h after administration except in liver and spleen as compared to administration of free doxorubicin (Van Hoesel 1984a).

In continuation of our previous studies we decided to investigate whether treatment with doxorubicin entrapped in negatively charged liposomes compared to treatment with free doxorubicin, has beneficial effects on white blood cell counts, differential counts, thrombocyte counts, hemoglobin levels, erythrocyte counts, reticulocyte counts, and bone marrow differential counts. In addition, doxorubicin levels in relevant cell populations in peripheral blood and bone marrow were assessed with laser flow cytometric techniques in order to correlate the findings with doxorubicin concentrations.

The study was performed with negatively charged liposomes and with dosage levels which have been shown in previous studies to result in adequate antitumor activity against the IgM immunocytoma in the LOU/M Wsl rat (Van Hoesel 1984; Van Hoesel 1984a).

MATERIALS AND METHODS

Animals. Breeding pairs of LOU/M Wsl rats were kindly provided by Dr.H.Bazin (Catholic University of Louvain, Brussels, Belgium). Animals were bred at the National Institute of Public Health and Environmental Hygiene. Male animals, weighing approximately 220 g and 12 weeks old, were used. The animals were housed in pairs in wire cages. Water and commercially available food (Muracon, Hope Farms, Woerden, The Netherlands) were provided ad libitum.

Drug preparation. Doxorubicin (Adriablastine^{R}, Roger Bellon S.A., Neuilly sur Seine, France) was obtained commercially. The lyophilized powder was reconstituted with sterile water to a concentration of 2.0 mg/ml. Negatively charged liposomes (egg-phosphatidylcholine: cholesterol: phosphahidylserine = 10:4:1) and doxorubicin entrapped in those liposomes were prepared according the procedure, described elsewhere (Van Hoesel 1984a).

Dosage schedule. The experiments were performed at two dosage levels: doxorubicin 1 mg/kg and 2 mg/kg. Injections were given on five consecutive days into the tail vein.

In the 1 mg/kg experiment the following groups have been investigated: the administration of saline 0.9% w/v; doxorubicin 1 mg/kg entrapped in negatively charged liposomes (lip^{-}DXR); and free doxorubicin 1 mg/kg (free DXR). On days 6, 9, 11, 13, 20, 34 and 48 after the start of treatment five animals of each group were killed.

In the 2 mg/kg experiment the following groups have been investigated: saline 0.9% w/v; empty negatively charged liposomes; doxorubicin 2 mg/kg entrapped in negatively charged liposomes; and 2 mg/kg free doxorubicin. On days 3, 6, 8, 10, 13 and 15 after the start of treatment four animals of each group were killed.

Section. During ethyl ether anaesthesia peripheral blood was obtained by orbital puncture and subsequently the animals were killed by exsanguination. Part of the blood was collected in EDTA-coated vials for determination of hemoglobin content, blood cell counts and peripheral smear. Another part of the blood was collected in freshly prepared ACD

buffer (sodium citrate 3.22 g/l; NaH_2PO_4 $2H_2O$ 3.40 g/l; Na_2HPO g/l; NaCl 4.96 g/l; glucose 4.02 g/l; citric acid 1.17 g/l) and kept at 0°C for laser flow cytometric analysis. Bone marrow was obtained by flushing both femora and collected in freshly prepared ACD buffer and stored at 0°C until laser flow cytometric analysis. Part of the bone marrow was prepared for differential counts.

Hematological parameters. Hemoglobin, mean corpuscular volume (MCV), erythrocyte count, and leukocyte count were measured with a Coulter Counter Model S5 (Coulter Electronics, Harperden, Herts, U.K.) after predilution in Isoton II (Coulter Electronics) 1:449,4. Thrombocyte counts were determined in a counting chamber after dilution in Trivinsol (RIVM) 1:200. Reticulocyte counts were determined on peripheral smears stained with fresh methylene blue counting 2000 erythrocytes. Differential counts of peripheral blood and bone marrow were made on smears stained in May Grünwald Giemsa. In peripheral smears 200 white cells were counted; in bone marrow smears 400 white cells on 3 slides, scoring the cells of the red series in the meanwhile.

Laser Flowcytometric (FCM) studies. With three parameter laser flowcytometry cellular DXR concentrations have been determined in hematopoietic and peripheral blood cells and their subpopulations on a System 50H Cytofluorography (Ortho Diagnostic Systems Inc. Westwood, MA, U.S.A.) equipped with a 5W argon laser (164-05, Spectra Physics, Mountain view, CA, USA) running at 488 nm, 0.5 W. DXR-fluorescence was measured with a barrier filter OG 550 (Melles Griot Optical Industries, Costa Mesa, CA, USA). Data on forward light scatter, perpendicular light scatter and fluorescence per cell were measured in area mode, stored in list mode, and analysed on a PDP 11/34 computer (Digital Electronic Company, Maynard, MA, USA). Mean cellular DXR concentrations are expressed in arbitrary fluorescence units/cell (FU/cell) after correction for endogenous fluorescence. HPLC analysis has been performed in order to correlate the arbitrary fluorescence unit with cellular drug concentration. As before, HPLC analysis has been shown to correlate well with the FCM determination of the cellular DXR concentration (Speth 1985). The materials and methods applied for FCM and PHLC have been described in detail in the latter study.

Hematologic parameters

Leukocyte counts are depicted in Chart 1. The control values of the 1 mg/kg and the 2 mg/kg experiments, which were not performed synchronously differ significantly from each other ($p < 0.01$). In addition, in the 1 mg/kg experiment the leukocyte counts in control animals drop spontaneously after day 20 ($p < 0.05$).

Compared to their own controls, the leukocyte counts are depressed in both experiments from the first day of measurement (i.e. day 6 and 3 for 1 and 2 mg/kg experiment respectively) until day 13. The administration of empty negatively charged liposomes had no effect on leukocyte counts.

At both dosage levels white blood cell counts recover simultaneously in the free DXR and lip⁻DXR treated groups.

The differential counts on peripheral smears showed that both the lymphoid and myeloid series were depressed (data not shown). In the 2 mg/kg experiment a drop of peripheral lymphocyte count in both treatment groups is already noticed on day 3, whereas the absolute number of granulocytes does not differ from control values on that day.

In bone marrow smears of the 2 mg/kg experiment the ratio between young myeloid cells and the total myeloid population equals on day 3 the ratio found in bone marrow smears of control animals (Chart 2). After day 3 the ratio declines with equal speed in both lip⁻DXR and free DXR treated groups. After day 8 the prevalence of young myeloid cells in the bone marrow rapidly increases.

Compared to control values the thrombocyte counts in both treatment groups show a slight drop in the 1 mg/kg experiment (Chart 3). After day 13 recovery begins. The 1 mg/kg free DXR treated animals show on days 20 and 34 an overshoot in thrombocyte counts. In the 2 mg/kg experiment a biologically relevant decrease of thrombocyte counts in both lip⁻DXR and free DXR treated groups is observed after day 6. The recovery of thrombocyte counts starts after day 13. Complete recovery has not yet been reached at the end of the experimental period.

In the 1 mg/kg experiment no change in hemoglobin levels is observed (Chart 4). In the 2 mg/kg experiment, however, the hemoglobin levels in both free DXR and lip⁻DXR treated groups decline after day 10. The findings for erythrocyte counts are the same as for hemoglobin levels (Chart 5). At

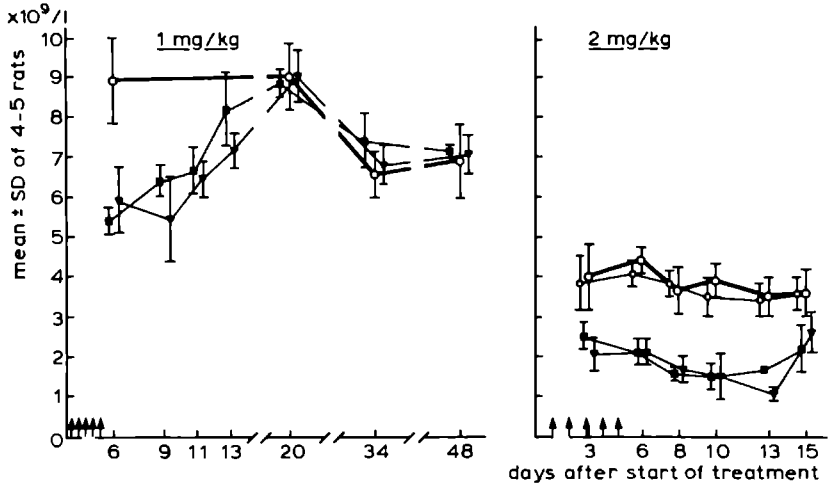

Chart 1. White blood cell counts

O : saline 0,9% w/v ↑ : i.v. injection

o : blank negatively charged liposomes

▼ : free DXR

■ : lip⁻DXR

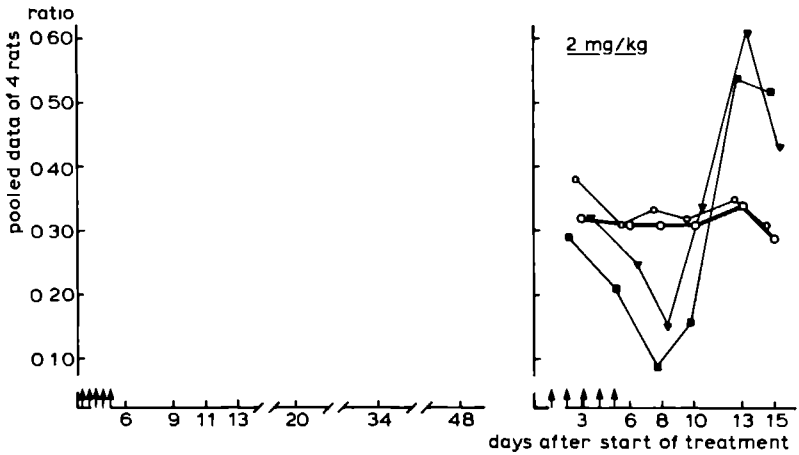

Chart 2. Ratio white blood cell precursors: total white blood cells in bone marrow

O : saline 0.9% w/v ↑ : i.v. injection

o : blank negatively charged liposomes

▼ : free DXR

■ : lip⁻DXR

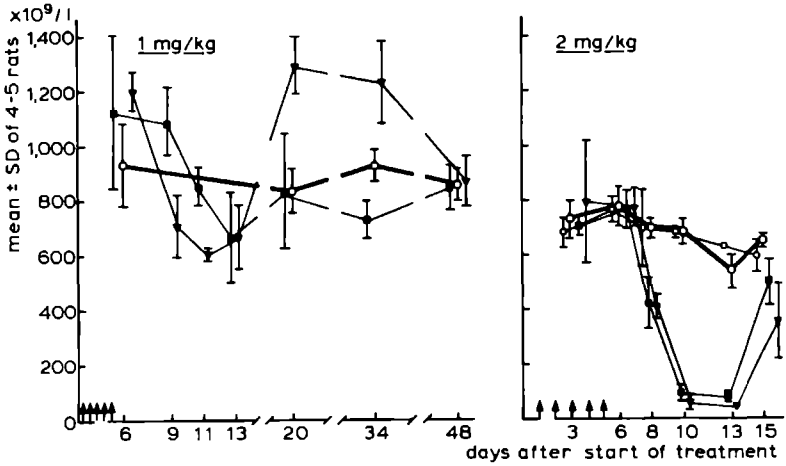

Chart 3. Thrombocyte counts

○ : saline 0.9% w/v

▲ : i.v. injection

ø : blank negatively charged liposomes

▼ : free DXR

■ : lip⁻DXR

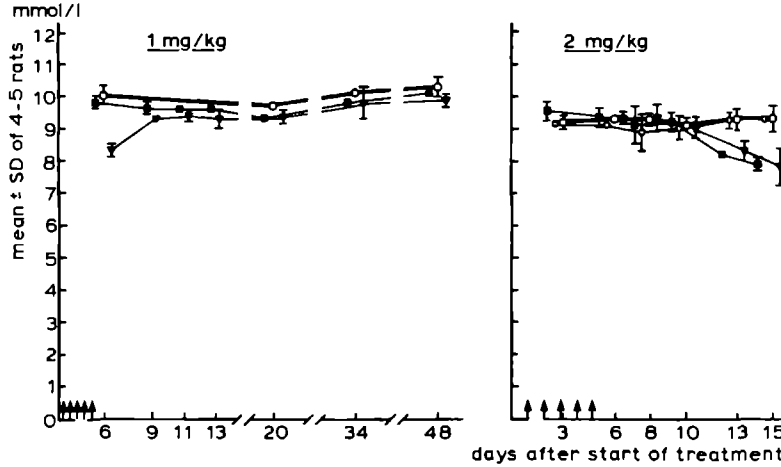

Chart 4. Hemoglobin levels

○ : saline 0.9% w/v

▲ : i.v. injection

o : blank negatively charged liposomes

▼ : free DXR

■ : lip⁻DXR

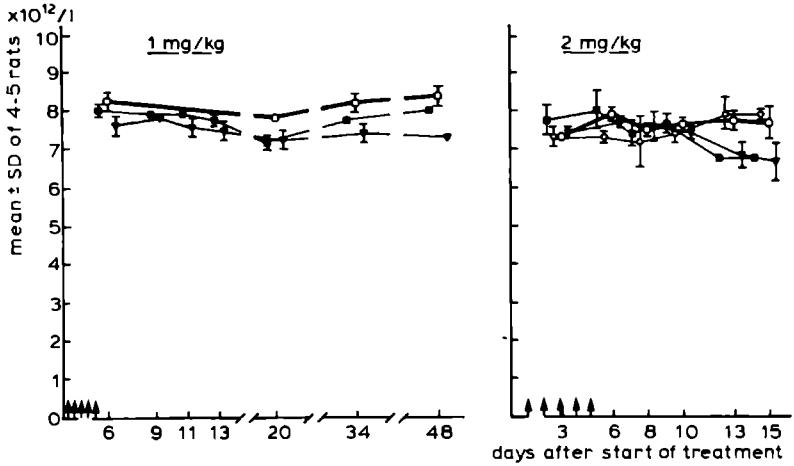

Chart 5. Erythrocyte counts

O : saline 0.9% w/v

o : blank negatively charged liposomes

▼ : free DXR

■ : lip⁻DXR

↑ : i.v. injection

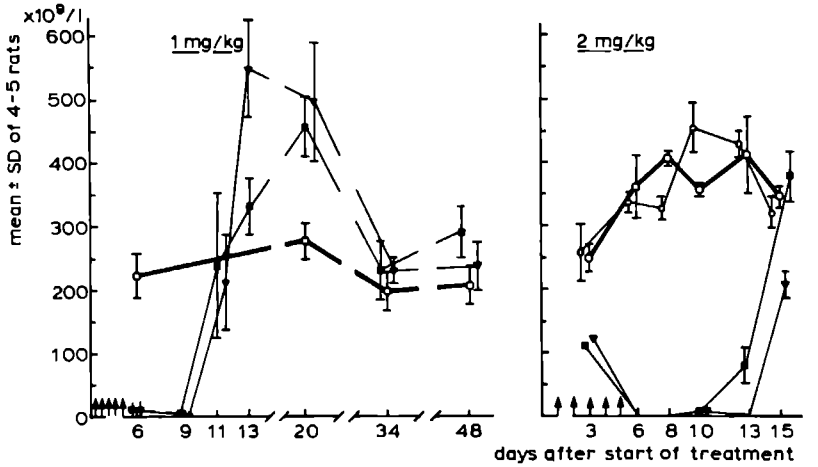

Chart 6. Reticulocyte counts

O : saline 0.9% w/v

o : blank negatively charged liposomes

▼ : free DXR

■ : lip⁻DXR

↑ : i.v. injection

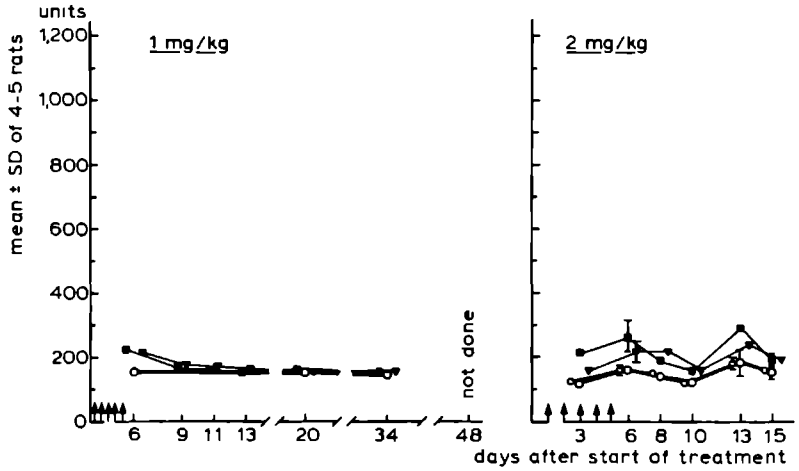

Chart 7. Mean cellular fluorescence in peripheral blood cells

O : saline 0.9% w/v ↑ : i.v. injection

o : blank negatively charged liposomes

▼ : free DXR

■ : lip⁻DXR

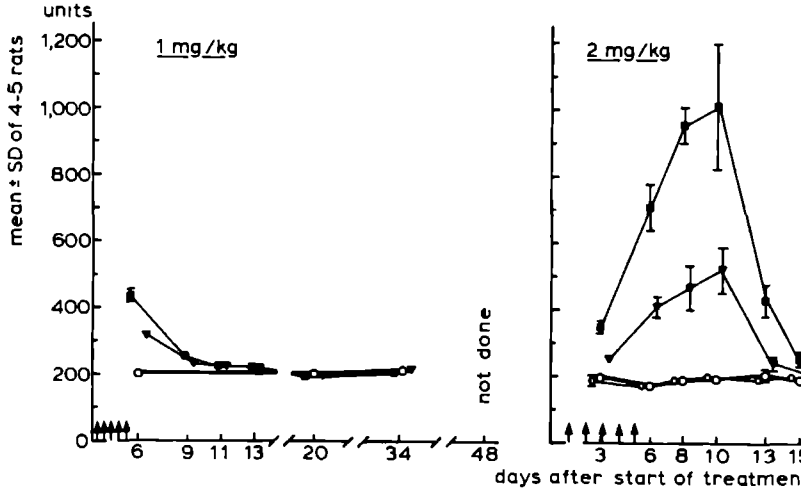

Chart 8. Mean cellular fluorescence in bone marrow cells

O : saline 0.9% w/v ↑ : i.v. injection

o : blank negatively charged liposomes

▼ : free DXR

■ : lip⁻DXR

day 48 in the 1 mg/kg experiment the erythrocyte count in lip⁻DXR treated rats differed from the erythrocyte count of free DXR treated animals at the $p < 0.001$ level, whereas the differences from control value were at the $p < 0.01$ and $p < 0.001$ level respectively. In the 2 mg/kg experiment at day 15 the erythrocyte count of lip⁻DXR treated animals differed at $p < 0.05$ level from control value. The drop in hemoglobin level and erythrocyte count of free DXR and lip⁻DXR treated animals is preceded by a total depression of reticulocytes in both treatment groups (Chart 6).

The MCV values remain constant during both experiments (data not shown).

Laser flow cytometric data

In peripheral white blood cells the fluorimetric determined cellular DXR concentration in both treatment groups at both dosage levels scarcely supercedes the endogenous fluorescence of controls during and after the treatment period (Chart 7).

In bone marrow at the 1 mg/kg dose level the fluorescence levels at day 6 are clearly higher than control values, whereas higher fluorescence levels are found in the group treated with lip⁻DXR (Chart 8). At day 9 for both free doxorubicin and liposomal entrapped doxorubicin cellular DXR concentration levels return to zero (fluorescence levels of controls). At the 2 mg/kg dose level a small further increase in cellular DXR concentration was observed in both treatment groups even after cessation of treatment. The highest levels are reached at day 8-10. The administration of lip⁻ DXR resulted in the highest levels. After day 10 a rapid decline is noticed resulting in fluorescence levels in the range of control values at day 15. The DXR related fluorescence is particularly found in myeloid cells (data not shown).

There are considerable differences in leukocyte control values in the two experiments. In addition, the leukocyte counts of the control groups at days 34 and 48 in the 1 mg/kg experiment show a statistical significant decrease. The differential counts demonstrate that the differences found in control values are mainly due to variations in lymphocyte counts, which cannot be explained (data not shown). For the reader it is important to know that the relative lymphocyte counts in rats are in the 75-90% range.

The differential bone marrow counts of the 2 mg/kg experiment are available. Except on day 6 in the free DXR treated group the contribution of the lymphocytic population to hematopoietic bone marrow cells is increased during bone marrow suppression. The observations with regard to peripheral lymphocytic counts, made in this study, cannot be explained, nor is the biological implication clear.

Entrapment of doxorubicin in negatively charged liposomes, did not result in a clear advantage compared to administration of free doxorubicin with regard to the degree of leukocytopenia nor the duration of it. Rahman et al. (1985) reported a smaller drop of the white blood cell count which occurred later than after administration of free drug after a single dose of doxorubicin entrapped in positively charged liposomes.

The thrombocytopenia appeared to be dose dependent, and was not affected by the method of doxorubicin administration.

Anemia did not occur during these experiments. Although at the end of the 2 mg/kg experiment some drop in hemoglobin level is observed, related to a precedent inhibition of erythropoiesis as appears from the depression of reticulocyte count, the biological relevance is doubtful. On day 15 the serum albumin level in the 2 mg/kg free DXR treated group had decreased to 6.7±0.6 g/l (n=4), as expression of the DXR induced nephrotic syndrome. The concomitant disturbance in the water balance might be responsible for the observed lower hemoglobin level. However, the serum albumin level in lip^- DXR 2 mg/kg treated group on day 15 is 25.0±3.2 g/l. (n=4), while Hb level does not differ from free DXR treated animals. The life span of the rat erythrocyte is estimated to be about 60 days (Schalm 1965). This means that in one day about 1.7% of erythrocytes are to be renewed. If the suppression of reticulocytes lasts about 7 days, as is observed in the 2 mg/kg experiment, the erythrocyte count and the hemoglobin level are expected to

decrease about 12%. This value is in agreement with the observations made. In the 1 and 2 mg/kg experiment reticulocyte counts are depressed in both lip⁻DXR and free DXR treated groups at least untill day 9. On day 11 the reticulocyte counts were in the range of control values. From day 13 till at least day 20 the reticulocyte counts in both treatment groups are about twice the control values, pointing at the activation of a compensatory mechanism. Overall toxicity at the 2 mg/kg dose level, administered on five consecutive days impedes longer observation. In the 1 mg/kg experiment, which allowed prolonged observation, no change in hemoglobin level occurs. The unchanged MCV values suggest that insertion of major parts of the liposomal membrane into the erythrocytes is less likely.

The data on cellular DXR concentration in bone marrow cells are very remarkable. In the 1 mg/kg experiment the anthracycline related fluorescence declined after cessation of treatment as expected. In contrast, in the 2 mg/kg experiment, both in the free DXR, but less than in the lip⁻DXR treated rat the fluorescence steadily increased after the cessation of treatment. This phenomenon might indicate continuous doxorubicin levels in serum. The difference between the 1 mg/kg and 2 mg/kg experiment might be explained by saturation phenomena in the mononuclear phagocytic system or a threshold for doxorubicin accumulation in bone marrow. The fluorescence levels in the lip⁻ DXR exceeded those of free DXR. This observation cannot be explained by differences in differential counts; no predominance of absolute numbers of phagocytic cells in bone marrow of free or liposome entrapped doxorubicin treated groups was found.

Although anthracycline related fluorescence in the 2 mg/kg experiment increased untill days 8-10, bone marrow and subsequently peripheral blood show signs of recovery from this time on. Therefore, the meaning of intracellular anthracycline related fluorescence is not clear. The FCM does not discern metabolites, nor binding products of doxorubicin, in which fluorescent characteristics are preserved. However HPLC analysis on bone marrow cells did not show intracellular doxorubicin derived metabolites (data not shown). Further, this technique does not make any discrimination between the various maturation stages of bone marrow subpopulations or localisation of fluorescence at subcellular sites. For example, it might be possible that doxorubicin still bound to phospholipid constituents is phagocytosed in the lysosomal apparatus of differentiated myeloid cells,

which have lost their mitotic capacity. In this - highly speculative - setting doxorubicin wouldn't do any harm.

In conclusion, the administration of doxorubicin entrapped in negatively charged liposomes, which had shown to result in beneficial effects on doxorubicin induced cardiomyopathy and nephropathy with preservation of antitumor activity, in the IgM immunocytoma bearing male Lou/M Wsl rat has no advantage with regard to doxorubicin induced suppression of bone marrow activity at the dose levels investigated.

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Chapter 9

Concluding remarks and perspectives

The entrapment of doxorubicin in negatively charged liposomes reduced cardiomyopathy and nephrotoxicity with preservation of its antitumor activity, whereas entrapment of doxorubicin in positively charged liposomes resulted in less antitumor activity than the free drug. Administration of doxorubicin in negatively charged liposomes did not result in an advantage with regard to bone marrow function. Entrapment of doxorubicin in negatively charged liposomes increases the therapeutic index by decreasing the toxic effects.

From the present study it is not clear whether the severity of cardiomyopathy in the groups treated with liposome entrapped doxorubicin after prolonged observation times will yet reach the severity observed in the free doxorubicin treated group. This item is currently under investigation.

The beneficiary effects are thought to be the result of prolonged doxorubicin levels, "infused" into plasma by liver and spleen, where doxorubicin encapsulating liposomes have been shown to be taken up preferentially.

Selective uptake of liposomes by tumor cells is less likely because this would require the passage of liposomes across endothelial linings. In tumor tissue four types of capillaries have been discerned: 1) continuous capillaries, lined by an uninterrupted sheet of endothelial cells joined by typical tight junctions and resting on a well-formed basement membrane; 2) discontinuous capillaries with an incomplete endothelial lining lying on a continuous or fragmented basement membrane; 3) sinusoidal blood channels; sometimes the endothelial lining is lost and blood spaces are lined by tumor cells 4) giant capillaries, with a lumen that may exceed 50 um in diameter and consisting of a single layer of endothelial cells with little of no connective tissue (Poste 1984). The unpredictable variations in amounts of normal and abnormal vasculature in tumor tissue makes direct contact between liposomal entrapped cytostatic drug and tumor cells less likely.

The preferential uptake of doxorubicin entrapped in liposomes in liver

and spleen has focused the attention to the mononuclear phagocytic system as a mediator in the cytostatic action of doxorubicin. The uptake of doxorubicin in macrophages has been shown to activitate them to cytotoxicity (Haskill, 1981, Martin 1982, Giavazzi 1984). Studies on this item performed by our cooperative groups have failed to show influx of macrophages or monocytes into the IgM immunocytoma as carriers for liposomal entrapped doxorubicin (Van Gessel, manuscript in preparation).

Long-term liposome related toxicity has not been considered in this thesis. In current literature no elaborate studies on this topic are available. The (repeated) administration of liposomes in equivalent amounts to the administration of fat emulsions as used in total parenteral nutrition would not be scientifically justifiable. Although liposomes are made of biodegradable constituents, toxicity is not for that reason precluded. Furthermore, Poste (1984) warns against the possible consequences of blockading the mononuclear phagocytic system, not only by overwhelming the phagocytic capacity, but also by the inhibitory effects of cytostatic drugs on nucleic acid and protein synthesis in phagocytes. Repeated liposome administration in mice has been demonstrated to blockade the reticuloendothelial system, the degree of blockade being related to liposome size and composition, size and frequency of liposome dose and the presence of lipid peroxides (Allen, 1984).

In order to explore the value of liposome entrapped cytostatic drugs for intraperitoneal administration and for targeting, a nude rat model inoculated intraperitoneally with an human ovarian cancer cell line is currently being developed at the Dutch National Institute of Public Health in collaboration with the Department of Cytology and Histology and the Department of Gynecology and Obstetrics (Catholic University Nijmegen). The efficacy of liposomes encapsulating doxorubicin, cisplatin, radiopharmaca, and/or monoclonal antibodies will be the objective of the investigations.

The application of liposome entrapped drugs will first have to prove their advantages compared to conventional drug administration in clinical medicine. However, several problems have yet to be solved before application on a large scale becomes feasible; e.g.

- development of techniques for production of liposomes at large scale,
- reproducibility of liposome size using large scale production methods,
- sterility, not only outside the liposomes, but also inside the liposome,
- shelf life of liposome preparation,

- stability of liposome suspensions (retention of entrapped drug, absence of aggregation and sedimentation),

and additional toxicity studies.

In case of studies in human beings, in addition to the standard requirements for the evaluation of toxicity of new drugs in cancer medicine, activation of the complement system and the development of an immune response should be surveyed.

Most importantly, even if a well known drug is incorporated in liposomes, the whole should be considered as a new drug.

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Doxorubicin is a cytostatic drug with a wide spectrum of antitumor activity. Its use in clinical practice is associated with a dose dependent incidence of doxorubicin induced cardiomyopathy. The studies described in this thesis are intended to investigate whether the administration of doxorubicin entrapped in liposomes results in less cardiomyopathy and bone marrow toxicity with preservation of antitumor activity in the IgM immunocytoma bearing LOU/M Wsl rat.

In chapter 1 an outline of the present investigation is given.

In Chapter 2 theoretical considerations on increasing the therapeutic index are given. The attention is focused on liposomes as drug carriers, which are defined as microscopic structures consisting of one or more concentric lipid bilayers enclosing an equal number of aqueous spaces. The historical background is depicted. The several types of liposomes are described. Some chemical and physical properties of liposomes are reviewed, and the methods of preparation of liposomes are briefly explained. Subsequently, the interactions of liposomes with plasma proteins, with cells in vitro, and the fate of liposomes in vivo are discussed. The incorporation of drugs into liposomes is briefly mentioned. The experimental results of entrapment of methotrexate, cytosine arabinoside, and actinomycin-D are reviewed. Some other, still speculative, applications of liposome entrapped non-cytostatic drugs are referred to.

In Chapter 3 the origin and chemistry of doxorubicin is discussed. The literature on analytical methods and pharmacokinetics is reviewed. The range of activity against human cancer is depicted. The binding of doxorubicin to DNA, the doxorubicin generation of free radical formation, and its metal chelation properties, in addition to its membrane binding capacity are explained and their relevance for antitumor activity and toxicity is discussed. The doxorubicin induced toxicity, and cardiotoxicity in particular, are reviewed. The lines of investigations on diminishing doxorubicin induced cardiomyopathy, which have emerged in the literature in the past decade, are summarized.

In chapter 4 a method for analyzing doxorubicin and doxorubicinol in micro volumes of rat plasma is described. The extraction of doxorubicin, doxorubicinol, and daunorubicin is performed with chloroform-1-heptanol (1 : 1) mixture. This organic solvent in a phase-volume ratio of 1 : 5 results in a 95% recovery of doxorubicin. Aglycone formation is prevented by using 0.2 M H_3PO_4 in the aqueous phase. The analysis is performed on high-performance liquid chromatographic equipment using the reversed-phase method. Daunorubicin is used as an internal standard. To circumvent the problems associated with the strong adsorptive effects of doxorubicin all glassware is silanized, and desipramine-HCl is added to the aqueous phase and the mobile phase. With this method the calibration curves for standard solutions of doxorubicin in water are linear over the concentration range from 0.1 μg/100 μl to 10 ug/100 ul and for standard solutions of doxorubicin in plasma over the concentration range 1 ng/100 μl plasma to 100 μg/100 μl plasma. Pharmacokinetic data, obtained with this method are given.

Antitumor activity, cardiotoxicity, and nephrotoxicity induced by doxorubicin in male LOU/M Wsl inbred rats, each bearing a transplantable solid IgM immunocytoma are described in Chapter 5. Inoculation with 1 x 10^4 IgM immunocytoma cells subcutaneously in the left flank resulted on day 18 after inoculation in tumors with a diameter of 15.8 ± 3.8 mm. Animals were treated with intravenous injections of doxorubicin (dose range 0.015 - 4.0 mg/kg body weight on five consecutive days, followed by 1 weekly injection for 7 weeks. Tumor regression was observed with 0.5 mg doxorubicin/kg. Complete disappearance of the tumor was induced with 1 mg doxorubicin/kg. Light microscopic evidence of cardiotoxicity scored as grade III was only observed at a dose of 1.0 mg doxorubicin/kg. Histologic evidence of renal damage, which resulted in albuminuria and very low serum albumin levels, was seen above a dose of 0.5 mg doxorubicin/kg. In the group that received 1.0 mg doxorubicin/kg the serum albumin level decreased from 33.6 ± 4.1 to 1.5 ± 0.5 g/liter. Ascites and hydrothorax were observed simultaneously. The same experiments have been performed with non-tumor-bearing rats, in which no major differences have been observed. In conclusion, doxorubicin induced antitumor activity, cardiotoxicity and nephrotoxicity can be studied simultaneously in the same LOU/M Wsl rat. Albuminuria due to renal damage led to extremely low serum albumin levels. Ascites and hydrothorax

are not necessarily a consequence of the observed cardiomyopathy.

Because severe doxorubicin induced nephropathy has been observed in the study described in the previous chapter, in Chapter 6 the question is raised whether nephropathy adds to, or even might be responsible for doxorubicin induced cardiomyopathy in rats. The temporal relationship between the onset of doxorubicin induced cardiomyopathy and nephropathy in male and female LOU/M Wsl rats has been studied. Modification of the treatment schedule did not circumvent nephrotoxicity. The 1 mg doxorubicin/kg dose, administered i.v. on five consecutive days and thereafter weekly reaching a cumulative dose of 13 mg doxorubicin/kg resulted in male LOU/M Wsl rats in albuminuria of more than 10 g/liter at day 14, whereas female rats only reached a similar level of albuminuria > 10 g/liter at day 49. As a consequence serum albumin levels in male LOU/M Wsl rats decreased to levels below 10 g/liter, while female rats maintained serum albumin levels till day 49. At day 10 in male and female rats a grade 1.0 - 1.5 cardiomyopathy score, assessed according to the modified Billingham scoring system, was found gradually increasing to grade 2.5 - 3.0 cardiomyopathy, both in males and females, on day 49. In male rats the nephropathy developed steadily from day 14 and thereafter, whereas in females the rate of development of kidney damage was slower and at the endpoint of the study the severity of kidney lesions was less in comparison to male rats. The onset of cardiomyopathy and nephropathy was simultaneously. In conclusion, the doxorubicin induced cardiomyopathy observed in LOU/M Wsl rats is a phenomenon which is independent of the nephropathy. This is in agreement with experience in clinical practice, where doxorubicin induced nephropathy is not observed in contrast to cardiomyopathy. Because both phenomena are independent, the nephrotic syndrome does not depreciate the value of the rat as a model for studies on doxorubicin induced cardiomyopathy.

In Chapter 7 the effects of entrapment of doxorubicin in liposomes on reduction of cardiotoxicity and nephrotoxicity with preservation of antitumor activity are described. Doxorubicin has been encapsulated in negatively charged liposomes (lip^- DXR) composed of egg phosphatidylcholine, cholesterol and phosphatidylserine and in positively charged liposomes (lip^+ DXR) composed of phosphatidylcholine, cholesterol

and stearylamine. Doxorubicin, lip$^-$ DXR, and lip$^+$ DXR have been adminstered i.v. (0, 0.25, 0.5, 1.0 and 2.0 mg/kg) on day 17 for 5 consecutive days and then weekly into IgM immunocytoma bearing male LOU/M Wsl rats. Control animals died of progressive tumor growth 27 days after inoculation. Antitumor activity was dose dependent for all three preparations. Free DXR and lip$^-$ DXR showed the same antitumor activity. Lip$^+$ DXR had less antitumor activity. The overall survival of tumor-bearing animals treated with 2.0 mg/kg lip$^-$ DXR was significantly prolonged ($p < 0.05$) in comparison to the animals treated with 2.0 mg/kg free DXR. Treatment with free DXR resulted after 47 days in grade III cardiomyopathy, whereas treatment with lip$^-$ DXR resulted in grade I cardiomyopathy. Compared to treatment with free DXR, treatment with DXR encapsulated in either type of liposome resulted in less kidney damage, less albuminuria and preservation of serum albumin levels. Treatment with liposomal entrapped doxorubicin resulted in lower tissue doxorubicin levels at 4 h and 24 h after one i.v. injection of 1 mg doxorubicin/kg when compared to free drug. Lip$^-$ DXR, however, resulted in high doxorubicin tissue levels in spleen and liver. It is concluded that treatment with lip$^-$ DXR or lip$^+$ DXR resulted in prolonged survival, less albuminuria, higher serum albumin levels, less lesions in heart and kidney, correlating with lower DXR levels in these organs. Only lip$^-$ DXR had the same antitumor effect as free DXR, resulting in a therapeutical advantage due to less toxicity.

Because bone marrow toxicity is the dose limiting toxicity in clinical practice, in Chapter 8 hematological parameters during and after treatment with lip$^-$ DXR, which had been shown to result in less toxicity in the studies described in the previous chapter, were compared to treatment with free DXR. It was concluded that administration of lip$^-$ DXR at the dose levels investigated did not result in an advantage with regard to extent and duration of suppresion of bone marrow activity. Bone marrow recovery set in despite increasing cellular doxorubicin levels; these latter were measured using laser flow cytometric techniques.

In Chapter 9 some questions about the applicability of liposomes as drug carriers in clinical medicine and lines for future research are discussed.

Doxorubicine is een stof die de celdeling remt (cytostaticum) met een breed werkingsspectrum. Het wordt daarom bij de behandeling van gezwelsziekten veelvuldig gebruikt. De toepassing ervan gaat gepaard met een met de totale dosis toenemende kans op beschadiging van de hartspier. Het onderzoek, dat in dit proefschrift beschreven wordt, heeft als belangrijkste doelstelling na te gaan of de toediening van doxorubicine ingebouwd in liposomen resulteert in minder beschadiging van de hartspier en remming van de activiteit van het beenmerg met behoud van antitumor werking. Voor dit doel werd de LOU/M Wsl rat, geënt met een IgM immunocytoma als model bruikt.

In hoofdstuk 1 wordt de opbouw van het onderzoek, dat ten grondslag ligt aan dit proefschrift, geschetst.

In hoofdstuk 2 wordt een theoretische beschouwing over het begrip therapeutische index gegeven. Vervolgens wordt de aandacht gericht op liposomen als dragers van geneesmiddelen. Liposomen zijn microscopische structuren, die uit een of meer concentrische bimoleculaire laagjes vet bestaan. Deze omsluiten een aantal ruimten gevuld met water, dat gelijk is aan het aantal membranen waaruit het liposoom bestaat. De historische achtergrond wordt geschetst. Een beschrijving van de verschillende typen liposomen wordt gegeven. Enige chemische en fysische eigenschappen van liposomen worden kort samengevat. De bereidingswijzen van liposomen worden beknopt uitgelegd. Vervolgens wordt de wisselwerking van liposomen met plasma-eiwitten en met cellen in de reageerbuis en het lot van liposomen in levende dieren besproken. Het concept om geneesmiddelen in te bouwen in liposomen wordt belicht. De experimentele ervaringen met cytostatica als methotrexaat, cytosine arabinoside en actinomycine - D worden gemeld. Er wordt verwezen naar speculatieve toepassingen van andere dan cytostatische geneesmiddelen ingebouwd in liposomen.

In hoofdstuk 3 wordt de herkomst en de chemische achtergrond van doxorubicine besproken. Gebaseerd op de literatuur wordt een overzicht van analytische methoden en farmakokinetiek gegeven. Het werkingsspectrum in de humane oncologie wordt geschilderd. Er wordt ingegaan op de binding van

doxorubicine aan DNA, op de door doxorubicine uitgelokte opwekking van vrije radicalen, op de eigenschap metalen te cheleren en op het vermogen zich aan membranen te binden en de betekenis hiervan voor antitumorwerking en toxiciteit. De door doxorubicine geinduceerde toxiciteit en de beschadiging van de hartspier in het bijzonder, worden besproken. De lijnen van onderzoek naar mogelijkheden om de beschadigende werking op de hartspier te verminderen worden samengevat.

In hoofdstuk 4 wordt een methode voor de meting van doxorubicine en doxorubicinol in microvolumes ratteplasma beschreven. De onttrekking van doxorubicine, doxorubicinol en daunorubicine wordt uitgevoerd met mengsel van chloroform-1-heptanol (1:1). Hiermee wordt in een verhouding onttrekkingsvloeistof : plasma = 5 : 1, 95% van de doxorubicine aan een monster onttrokken. De vorming van aglyconen wordt voorkomen door 0.2 M H_3PO_4 in de waterfase te gebruiken. De analyse wordt uitgevoerd op een hoge druk vloeistof chromatograaf, gebruik makend van de "reversed phase" methode. Als interne standaard wordt daunorubicine gebruikt. Om adsorptie van doxorubicine te vermijden wordt het laboratorium-hulpmateriaal voorbewerkt met 2% trimethylchlorosilaan in tolueen en wordt desipramine-HCl aan de waterfase en het loopmiddel toegevoegd. De toepassing van voornoemde methode resulteert in ijklijnen voor oplossingen doxorubicine in water, die lineair zijn in het traject van 1 ng/ml tot 100 µg/ml. Voor doxorubicine in plasma over een traject van 10 ng/ml tot 1 mg/ml. De resultaten van onderzoek naar farmakokinetische parameters, verkregen met deze methode

De antitumor werking, de beschadiging van hartspier en nier teweeggebracht door doxorubicine in mannelijke LOU/M Wsl ratten, geënt met een als een solide tumor op te vatten IgM immunocytoom, worden beschreven in hoofdstuk 5. Enting met 1 x 10^4 IgM immunocytoma cellen onderhuids op de linkerflank leidt op dag 18 na inoculatie tot een tumor met een diameter van 15.8 ± 3.8 mm. Aan de dieren wordt vervolgens doxorubicine intraveneus toegediend (dosering opklimmend van 0.015-4.0 mg/kg lichaamsgewicht) op 5 achtereenvolgende dagen, gevolgd door een toediening per week gedurende 7 weken. Afneming van tumor wordt gezien bij een dosis van 0.5 mg/kg. Verdwijning van tumor wordt waargenomen bij een dosis van 1 mg/kg. Met de lichtmicroscoop wordt graad III beschadiging van de hartspier vastgesteld

bij ratten behandeld met 1 mg/kg lichaamsgewicht. Histologische tekenen van nierbeschadiging, leidend tot albuminurie en zeer lage serum albuminespiegels worden waargenomen boven een dosis van 0.5 mg/kg. In de groep ratten, behandeld met 1.0 mg/kg nam de serum albumine concentratie af van 33.6 ± 4.1 g/l tot 1.5 ± 0.5 g/l. Ascites en hydrothorax treden gelijktijdig op. In niet-tumordragende dieren worden dezelfde resultaten verkregen. Geconcludeerd wordt dat cytostatische aktiviteit en beschadiging van hartspier en nier gelijktijdig in de LOU/M Wsl rat kunnen worden bestudeerd. Voorts wordt vastgesteld dat de ascites en hydrothorax meer waarschijnlijk het gevolg zijn van de zeer lage serum albumine concentraties dan van het falen van hartspierwerking.

In hoofdstuk 6 wordt ingegaan op de vraag of de nierbeschadiging bijdraagt of zelfs verantwoordelijk is voor de beschadiging van de hartspier bij ratten. Het ontstaan van door doxorubicine veroorzaakte beschadiging van hartspierbeschadiging en nierbeschadiging wordt in de tijd bestudeerd. Het onderzoek wordt zowel bij mannelijke als vrouwelijke ratten uitgevoerd, omdat in voorbereidende studies gebleken was dat er een geslachtsgebonden verschil in gevoeligheid voor door doxorubicine veroorzaakte toxiciteit is. In overeenstemming met de ervaringen in de kliniek, waar door doxorubicine veroorzaakte nierbeschadiging niet wordt waargenomen, levert dit onderzoek geen aanknopingspunt voor een oorzakelijk verband tussen nierbeschadiging en hartspierbeschadiging, omdat de nierbeschadiging en hartspierbeschadiging gelijktijdig tot ontwikkeling komen en vooral omdat de nierbeschadiging bij mannelijke ratten ernstiger is dan bij vrouwelijke dieren, terwijl de beschadiging van de hartspier voor beide geslachten zich gelijkelijk ontwikkelt.

In hoofdstuk 7 worden de resultaten van behandeling met doxorubicine ingebouwd in negatief geladen liposomen samengesteld uit ei-phosphatidylcholine, cholesterol en phosphatidyl serine en van doxorubicine ingebouwd in positief geladen liposomen samengesteld uit phosphatidylcholine, cholesterol en stearylamine beschreven. Onderzocht in de mannelijke LOU/M Wsl rat, geënt met het IgM immunocytoom blijkt doxorubicine in negatief geladen liposomen een antitumor werking te hebben die niet verschilt van vrij doxorubicine, terwijl de antitumor activiteit van doxorubicine in positief geladen liposomen hierbij ten achter blijft.

Op dag 47 van de behandeling leidt behandeling met vrij doxorubicine (1 mg/kg) tot graad III beschadiging van de hartspier, terwijl behandeling met doxorubicine (1 mg/kg) ingebouwd in negatief geladen liposomen de hartspierbeschadiging beperkt tot graad I. Wat betreft nierbeschadiging, albuminurie en serum albuminespiegel bieden beide typen liposomen vergelijkbare voordelen. Metingen van doxorubicine concentraties in plasma en weefsels tonen 4 en 24 uur na een eenmalige intraveneuze injectie van doxorubicine ingebouwd in liposomen lagere gehaltes in weefsels aan, met name in hart en nier. Toediening van doxorubicine in negatief geladen liposomen echter geeft in lever en milt hoge gehaltes. Omdat doxorubicine ingebouwd in negatief geladen liposomen resulteert in minder bijwerkingen en gelijkblijvende antitumor werking, vergeleken met behandeling met vrij doxorubicine, kan men besluiten dat behandeling met doxorubicine ingebouwd in voornoemde liposomen tot een therapeutisch voordeel leidt.

In hoofdstuk 8 wordt het effekt van toediening van doxorubicine ingebouwd in negatief geladen liposomen op hematologische parameters vergeleken met het effekt van vrij doxorubicine. Bij de onderzochte doseringen en toedieningsschema, heeft de eerstgenoemde toedieningsvorm geen kortere of minder ernstige beenmergremming tot gevolg. Het herstel van de aktiviteit van het beenmerg treedt in, hoewel cellulaire doxorubicine concentraties, gemeten met de laser flowcytometer, in beenmergcellen toenemen.

In hoofdstuk 9 worden de problemen, die nog opgelost moeten worden, alvorens liposomen als dragers van geneesmiddelen, op grotere schaal op hun waarde voor klinische toepassing kunnen worden getoetst, besproken. Voorts worden nieuwe lijnen van onderzoek besproken.

Dankwoord

De schrijver van dit proefschrift dankt de Direktie van het Rijksinstituut voor Volksgezondheid en Milieuhygiene voor de personele en materiele faciliteiten, die het hem mogelijk hebben gemaakt dit proefschrift te bewerken.

Prof.Dr.H.M.Pinedo heeft mij gestimuleerd onderzoek naar de door doxorubicine geinduceerde cardiomyopathie te ondernemen.

Peter Steerenberg heeft een zeer belangrijke steen aan het onderzoek bijgedragen. Zonder zijn dagelijkse zorg voor de voortgang van de experimenten zou dit onderzoek niet tot stand gekomen zijn.

Dr.J.G.Vos, Hoofd van het Laboratorium voor Pathologie, dank ik voor de begeleiding bij de interpretatie van het histologisch materiaal.

De Hooggeleerde Prof.Dr.D.J.A.Crommelin, Hoogleraar aan de Subfaculteit der Farmacie van de Rijksuniversiteit Utrecht en zijn medewerkers Louis van Bloois en Gert Storm ben ik dankbaar voor de ontwikkeling en bereiding van de in dit onderzoek toegepaste liposomen.

Het Hoofd van het Laboratorium voor Farmacologie, Dr.F.C.Hillen ben ik erkentelijk voor zijn coordinerende werkzaamheden. Dr.D.J. de Wildt heeft parallel aan onze onderzoekingen de funktie van het myocard van met doxorubicine behandelde dieren onderzocht.

Dr.J.A.M.A.Dormans verzorgde de electronenmicroscopie en de interpretatie ervan.

De Heer W.Kruizinga met zijn medewerkers Ben van Rheenen, Piet van Schaaik en Hans Strootman zorgden voor de biotechnische ondersteuning van de experimenten. Bovendien tekende hij het merendeel van de grafieken en hielp met de statistische berekeningen. De eerste experimenten die de basis hebben gelegd voor het verdere werk werden uitgevoerd in de carré waar Jan Smits de scepter zwaait.

Drs.B.C.Kruijt dank ik voor het kweken van de LOU M/Wsl rat.

Piet Ursem heeft de inoculaten verzorgd. Hand- en spandiensten werden verleend door Anneke Elgersma, Dirk de Geus, Arja de Klerk, Bert Verlaan en Jan Wilschut.

Nadat Coen Moolenbeek de obdukties op zeer nauwkeurige wijze had verricht, hebben Klaas de Jong, Ciska de Vlugt en Joke Robinson het materiaal met de methacrylaattechniek tot zeer fraaie coupes weten te bewerken.

Dr.J.C.Koedam, Hoofd van het Laboratorium voor Klinische Chemie en Hematologie, en zijn medewerkers ben ik erkentelijk voor de ontwikkeling van de albumine bepaling in ratteserum en de vele biochemische bepalingen. Mevrouw Drs.A.M.Hagenaars dank voor de immunologische bepalingen.

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Ingewijden weten welk een immens werk ten grondslag ligt aan tabel 5 in hoofdstuk 7. Het opwerken van plasma en weefsels werd uitgevoerd door Drs.A. van Dijk van de Afdeling Klinische Farmacie (Hoofd Drs.J.H.Glerum) van het Academisch Ziekenhuis Utrecht en zijn naaste medewerker Simon Klein. Hun beider inspanning heeft hoofdstuk 7 de nodige diepgang gegeven.

Ria Struys, daarbij tijdelijk bijgestaan door Marja Steeman, heeft met zichtbaar plezier in het werk het manuscript verzorgd. Het uitwerken van de geschreven tekst en van de aangebrachte verbeteringen liep op rolletjes.

Collega R.S.Kirk, patholoog-anatoom te Arnhem ben ik erkentelijk voor de korrekties in het Engels idioom, die hij zeer snel en op charmante wijze heeft aangebracht. Wim de Jong dank ik voor het kritisch volgen van de experimenten en het doorlezen van het manuscript.

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Dankzij de medewerking van vele collegae heb ik het werk in het laboratorium met de klinische werkzaamheden kunnen combineren.

Curriculum vitae

De schrijver van dit proefschrift werd geboren op 2 mei 1950 te 's-Hertogenbosch. Na de lagere school volgde hij de gymnasium B opleiding aan het St.Janslyceum aldaar. Het eindexamen werd in 1968 afgelegd. In hetzelfde jaar begon hij de studie Geneeskunde aan de Rijksuniversiteit te Utrecht. Het artsexamen werd op 31 januari 1975 afgelegd. De militaire dienstplicht vervulde hij als reserve 1e luitenantarts bij de Geneeskundige Compagnie van de 13e Brigade te Oirschot. Op 1 mei 1976 begon hij de opleiding tot internist aan de afdeling Inwendige Geneeskunde (Opleider: Dr.J.A.Flendrig) van het Catharina Ziekenhuis te Eindhoven. Vanaf 1 juli 1978 werd de opleiding voortgezet aan de Afdeling Inwendige Geneeskunde van het Academisch Ziekenhuis te Utrecht (Hoofden: Prof.Dr.A. Struyvenberg en Prof.Dr.J. van der Sluys Veer). Hij werd als internist ingeschreven in het Specialistenregister op 1 mei 1981. Vanaf die datum was hij als Fellow van het Koningin Wilhelmina Fonds verbonden aan het Antoni van Leeuwenhoekhuis te Amsterdam. Sedert 1 mei 1983 is hij werkzaam als wetenschappelijk ambtenaar bij de Afdeling Medische Oncologie (Hoofd: Prof.Dr.D.J.Th.Wagener) van de Kliniek voor Inwendige Geneeskunde (Hoofd: Prof.Dr.A. van 't Laar) van het St.Radboud Ziekenhuis te Nijmegen. Hij is als consulent Medische Oncologie verbonden aan het Integraal Kankercentrum Oost en als zodanig werkzaam voor het Integraal Kankercentrum Zuid.

Hij is gehuwd met Louise J.M. Suyskens. Zij hebben twee kinderen.

STELLINGEN

behorende bij het proefschrift van

Q G C M van Hoesel

I

De met een IgM immunocytoom geïnoculeerde Lou M/Wsl rat maakt gelijktijdige bestudering van antitumor werking en toxiciteit van cytostatica mogelijk.

Dit proefschrift.

II

Anthracyclines brengen in een aantal species nephropathie, albuminurie en hypoalbuminaemie teweeg. Daarom zijn ascites en hydrothorax in het proefdiermodel niet noodzakelijkerwijs uiting van cardiomyopathie.

Dit proefschrift.

III

Behandeling van een IgM immunocytoomdragende Lou M/Wsl rat met doxorubicine ingebouwd in negatief geladen liposomen resulteert in antitumoractiviteit, die vergelijkbaar is met conventionele behandeling met doxorubicine, maar heeft minder beschadiging van de hartspier en nier ten gevolge.

Dit proefschrift.

IV

De toediening van doxorubicine ingebouwd in liposomen resulteert in een wezenlijk andere verdeling van doxorubicine in weefsels, vergeleken met de conventionele toediening van doxorubicine.

Dit proefschrift.

V

De duur en de ernst van de remming van de activiteit van het beenmerg in de Lou M/Wsl rat wordt door toediening van doxorubicine ingebouwd in negatief geladen liposomen niet voordelig beinvloed in vergelijking met de conventionele toediening van doxorubicine.

Dit proefschrift.

VI

De toxiciteit en het uitblijven van verlenging van de overlevingsduur zijn argumenten tegen de aanbeveling van de Gastrointestinal Tumor Study Group om patienten met een rectumcarcinoom, dat door de wand van het rectum is gegroeid en/of aanleiding heeft gegeven tot lymfkliermetastasen, behalve met uitwendige bestraling, ook met 5-fluorouracil en semustine te behandelen.

GITSG N Engl J Med 1985; 312: 1465
Editorial N Engl J Med 1985; 312: 1512

VII

Een instrument om de uitwerking van de consultverlening in het kader van de integrale kankercentra op de kwaliteit van de patientenzorg te meten ontbreekt. Feitelijk is een prospectief vergelijkend onderzoek niet mogelijk.

VIII

Na chemotherapie levert toevoeging van bestraling van de primaire lokalisatie van een kleincellig anaplastisch bronchuscarcinoom geen wezenlijke bijdrage aan het resultaat van de behandeling.

Souhami et al. Br Med J 1984; 288: 1643

IX

Het meten van thyreoglobulinespiegels na verwijdering van de schildklier waarin zich een gedifferentieerd schildkliercarcinoom bevindt, levert een bijdrage aan de vaststelling van residu tumor of metastasen en behoeft niet in hypothyreote toestand plaats te vinden.

J.H.Bolk et al. Neth J Med 1985; 28: 340

X

Het aantal varianten van de klassificatie van colon- en rectumcarcinomen volgens Dukes is zodanig groot dat toepassing van de hierop gebaseerde klassificatie vaker leidt tot misverstand dan tot een ondubbelzinnige samenvatting van de anatomische uitbreiding van de tumor. De TNM klassificatie verdient de voorkeur.

XI

Het peil waarop in Nederland in centra patienten met testiscarcinoom behandeld worden, biedt voldoende infrastructuur om een klinisch onderzoek naar de waarde van het zogenaamde "wait and see" beleid bij klinisch stadium I testiscarcinoom te rechtvaardigen, mits het accent meer op "see" dan op "wait" ligt.

XII

De bovenbuik bevindt zich boven de onderbuik en maakt in gelijke mate deel uit van de linker en van de rechter buik.

Q.G.C.M. van Hoesel

Nijmegen 8 november 1985